Development of an intracellular glycolytic flux sensor for high throughput applications in E.coli

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Preface

The following PhD thesis was conducted in the period of the 1st of May 2013 until the 31st of May 2016. I, Christina Lehning, performed all experimental work. The data analysis was carried out in collaboration with my colleagues.
The PhD was funded by the European Union through the "Bactory" Marie Curie Fellowship.
The supervisor for the PhD study was professor Morten Otto Alexander Sommer and co-supervisor was professor Alex Toftgaard Nielsen. Their affiliation was Novo Nordisk Foundation Center for Biosustainability, DTU.

The cover and the chapter illustrations are made in collaboration with Denis Carrara.

Christina Eva Lehning, 19th November 2016

Novo Nordisk Foundation Center for Biosustainability, DTU, Hørsholm

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Table of content

Dansk abstrakt

Formålet med dette ph.d.-projekt var at konstruere, afprøve og anvende en intracellulært, vækst-uafhængig og direkte kvantificerbar glykolytisk flux biosensor i *E. coli*.

Kendskab til det metabolske flux i bakterieceller er af stigende betydning, da det er afgørende for den bakterielle fysiologi. Endvidere, vil viden om det glykolytiske flux kunne forbedre *in silico* modellering og metabolic engineering.

Den udviklede biosensor er baseret på transkriptionsfaktoren Cra og forbinder det metaboliske flux til ekspressionen af grønt fluorescerende protein (GFP). Det dynamiske område af den konstrueret biosensor dækker alle de glycolytiske flux der kan fremkaldes af forskellige kulstofkilder. Derudover er det også vist, at den er i stand til at monitorere en yderligere flux stigning.

Sensoren blev anvendt til at studere flux-ændring som følge af gen-knockouts i *E. coli* på enkelt-celle niveau. Dette var muligt ved hjælp af et paralleliseret high-throughput assay. Efter vækst i flere generationer i næringsrige- og minimale medier, kunne 2126 gen-knockouts, primært uden for kernen stofskifte processer, screenes. 3 gen-knockouts med en høj flux og 158 med en lav flux fænotype blev fundet, disse omfatter mange flageller og fag-gener samt mange hidtil ukarakteriserede proteiner.

Den udviklede glycolytiske flux biosensor tilbyder et værktøj til at screene for metaboliske flux ændringer på en effektiv, hurtig og parallelisebar måde. Anvendelsen af nye screeningstilgange kan forbedre vores forståelse af mikrobiel fysiologi og kan benyttes til at forbedre mikrobielle cellefabrikker.

English abstract

The aim of this PhD project was to construct, test and apply an intracellular, growth-independent and direct measureable glycolytic flux biosensor in *E. coli*.

Studying the metabolic flux of bacterial cells is of growing interest as it is of fundamental importance to bacterial physiology as well as for *in silico* modeling and metabolic engineering. The metabolic flux contains information about how efficiently a bacterium can utilize a given carbon source and in which extend it is directed towards the different central metabolic pathways. The knowledge of these fluxes can contribute to the development of efficient production pathways and the identification of possible accumulation points in the engineered pathway. Furthermore it can give information about regulatory networks within the cell.

The developed biosensor is based on the transcription factor Cra and links the metabolic flux to the expression of green fluorescent protein (GFP). The dynamic range of the final biosensor construct covers the whole range of natural, intracellular glycolytic fluxes, induced by different carbon sources and it could also be shown that it is even capable of monitoring a further flux increase.

The sensor was applied to study the flux-altering effects of gene knockouts in *E. coli* at the single cell level in a vastly parallelized and high-throughput manner. After growth for several generations in rich and minimal media, 2126 gene knockouts, mainly outside of the core metabolism, could be screened. 3 gene knockouts with a high flux and 158 with a low flux phenotype were found, comprising many flagella and phage related genes as well as many so far uncharacterized proteins.

Taken together, the glycolytic flux biosensor offers a tool to screen for metabolic flux changes in an efficient, fast and parallelizable way, opening up for novel screening approaches that enhance our understanding of microbial physiology and can be applied to improve microbial cell factories.

Outline of the thesis

Chapter 1 – Introduction

The introduction focuses on the development and application of different kinds of biosensors. In regards of the subject of the PhD thesis, there is an emphasis on transcription factor based biosensors, in particular the catabolite activator/repressor (Cra), and their application in metabolic engineering.

Chapter 2 – Biosensor development

This chapter presents the development of the Cra based glycolytic flux biosensor, which enables the measurement of the glycolytic flux in single cells during growth.

Chapter 3 – Biosensor application

This chapter presents the application study of the Cra biosensor. It is showing the example of a highly parallelized screen for the physiological effects on the glycolytic flux for all possible knockout mutants in the *E. coli* genome.

Chapter 4 – Conclusion and future perspectives

The last section of this PhD thesis is dedicated to discuss the placement of the findings of this study in the current scientific field and a further outlook.

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CHAPTER 1 Introduction

1.1 Biosensors

The use of biological systems to sense for environmental changes, desired products or dangers has a long tradition in human history. An early example are canary birds that were used to sense for toxic gases in mines, but still today pigs are used to search for truffles and snoop or rescue dogs to find landmines, drugs or buried humans (Slomovic *et al*, 2015). In all these cases, humans took and take advantage of traits of different organism, to detect signals they cannot detect with their own senses.

Modern research on biosensors follows a similar approach in order to find new solutions for medical and environmental diagnostics (Slomovic *et al*, 2015), biotechnological developments or the *in vivo* study of biological processes that were not measurable until now. Many biosensors are taking advantage of sensors or sensing principles that already exist in nature. The term biosensor is used for a range of different biological sensors, including whole-cells, proteins and RNA molecules. The sensors are then further adjusted to fit the purpose and coupled to a detectable readout. The term biosensor is generally not reserved for a specific tool or application but is applied for a wide and diverse range of possible sensors, readouts and applications.

Even though this study will focus on genetically encoded biosensors, especially those based on transcription factors (TF), a view highlight of non-genetically encoded biosensors is presented here in order to give an idea of the variety of different biosensors and their applications that were developed in the recent years.

Biosensors range from nano-bodies that can enable time-resolved studies of cellular responses *in vivo* on a molecular scale (Irannejad *et al*, 2013) to whole organism that promise to be used as living diagnostics within a patient (Kotula *et al*, 2014). Some biosensors are wired into an electronic circuit in order to directly convert the detected signal to an electronic impulse (Das *et al*, 2015). In other cases, the detection of the signal triggers a biological reaction, that can be subsequently measured, for example the expression of fluorescent markers. Biosensors often allow the *in vivo* study of cellular processes that were until then impossible to detect. An example is the recent study of Fosque *et al*, 2015. They applied a Ca^{2+}-sensitive, fluorescence-based biosensor to enable the real-time, *in vivo* study of how behavior change manifests in brain cells. Without the use of a biosensor, an *in vivo* real-time study was entirely impossible, and conclusions had to be drawn from samples that were taken long after

the trigger was applied, making them by far less meaningful or reliable. Even though numerous, different biosensors have been published in the recent years, the development of new biosensors can still be time consuming and laborious.

1.1.1 The concept of genetically encoded biosensors

Recent developments in synthetic biology allow the affordable construction of diverse, engineered cell libraries (Goodman *et al*, 2013; Kosuri & Church, 2014; Cavaleiro *et al*, 2015; Gibson, 2014; Bonde *et al*, 2015; Jiang *et al*, 2013). These capabilities enable a deeper understandings of biological processes and regulation (Bonde *et al*, 2016; Kosuri *et al*, 2013), as well as facilitate more rapid and efficient cell factory and protein engineering (Wang *et al*, 2009). Similarly, inexpensive deep sequencing simplifies the identification of beneficial genetic variants, often by multiplexing (Kosuri *et al*, 2013). This also enables rapid identification of specific genetic changes in microorganism, which evolutionary adopted to a defined environment as in adaptive laboratory evolution (ALE) experiments (Portnoy *et al*, 2011; LaCroix *et al*, 2015). Nevertheless, the development of new biotechnologically relevant production pathways is still challenging. The modern research is confronted with the task to identify those candidates out of the abundance of different variants that have a desired characteristic. In certain case, as for example the expression of a colored compound, the identification can be straight forward, but in many cases it is more challenging.

Genetically encoded biosensors enable to link the expression of a reporter molecule to the concentration of a certain molecule or other environmental trigger. By coupling the intracellular concentration of a small molecule to the read out of a fluorescent protein, differences in intracellular concentrations can be easily identified at the single cell level (Binder *et al*, 2012a). Biosensors have been applied in several high-throughput screens proving their relevance to enzyme engineering and cell factory optimization (Binder *et al*, 2013; Mustafi *et al*, 2012; Michener *et al*, 2012; Schendzielorz *et al*, 2014; Siedler *et al*, 2014b; Raman *et al*, 2014; Taylor *et al*, 2015). In Table I, a selection of highly relevant and successfully applied biosensors is listed, sorted by the kind of application. As this table shows, there have already been a number of different areas of application for biosensors from the screening for improved enzymes (Siedler *et al*, 2014a; Tang *et al*, 2013; Binder *et al*, 2012b; Schendzielorz *et al*, 2014) or production pathways (Tang & Cirino, 2011; Dietrich et al, 2013) from a synthetically generated library or a pool of evolutionary adapted variants (Chou et al, 2013;

Mahr et al, 2015; Yang et al, 2013). It has also been shown that biosensors can be applied to identify novel enzymes with a desired function from for example metagenomic libraries (Uchiyama & Miyazaki, 2010; Genee et al, 2016). One of the most promising advances in biosensor design for biotechnology is the successful development of dynamic pathway controls. These synthetic fed forward or negative feedback loops enable the dynamic auto-tuning of a pathway in response to the actual intracellular concentration of a precursor or for example a toxic intermediate (Zhang et al, 2012; Liu et al, 2015; Xu et al, 2014).

Table I) **A list of highlights of successfully applied biosensors for different purposes.** Proof of principle studies are excluded from this table but mentioned in the further chapters.

Sensed molecule	Sensor	Output	Reference
Improving enzymes			
NADPH	SoxR	eYFP	(Siedler *et al*, 2014a)
Triacetic acid lactone (TAL)	AraC	LacZ	(Tang *et al*, 2013)
L-Arg/L-Lys/L-His	LysG	eYFP	(Binder *et al*, 2012b; Schendzielorz *et al*, 2014)
Identification novel enzymes			
Benzoate	BenR	GFP	(Uchiyama & Miyazaki, 2010)
TPP (thiamine pyrophosphate); xanthine	ThiM19 riboswitch	Antibiotic resistance	(Genee et al, 2016)
Pathway optimization			
Mevalonate	AraC	LacZ	(Tang & Cirino, 2011)
Butanol	BmoR	TetR-GFP	(Dietrich et al, 2013)
Dynamic pathway control			
Acetyl-CoA	FadR	Synthetic fed forward activation	(Zhang *et al*, 2012)
Malonyl-CoA	FapR	Synthetic negative feedback loop	(Liu *et al*, 2015; Xu *et al*, 2014)
Improving adaptive evolution			
IPP (isopentenyl diphosphate)	Ing/AraC chimera	RFP	(Chou *et al*, 2013)
L-Lys	Lrp	eYFP	(Mahr *et al*, 2015)
L-Lys	Lys riboswitch	TetA	(Yang *et al*, 2013)

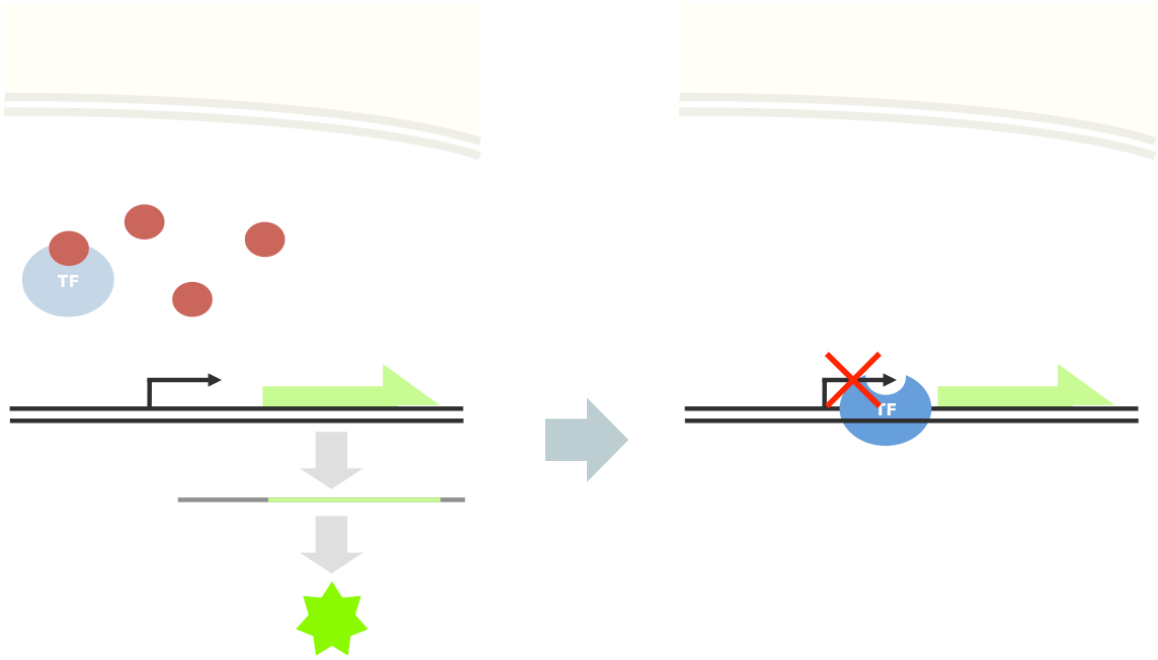
1.1.2 Output systems

The genetically encoded biosensors offer a modular output system that can be changed to meet the requirements of the application.

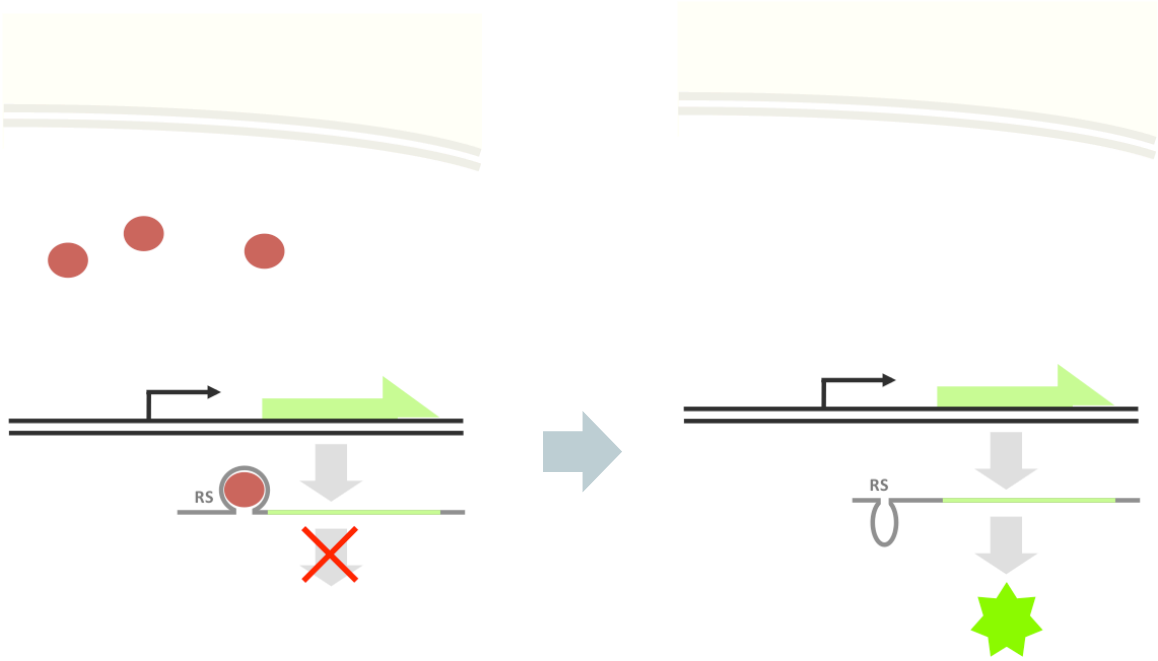
The most commonly used output is fluorescence. The expression of a fluorescent protein like GFP (Prasher *et al*, 1992) is controlled by the biosensor. Since it is established that there is a close correlation between the GFP expression and the fluorescent signal (Albano *et al*), the signal can be used as a reporter for the biosensor activity. Different instruments offer the option to screen for fluorescence, enabling many different screening methods, e.g. fluorescence microscopy, plate reader analysis, microfluidics or flow cytometry/FACS. These methods offer different resolutions, throughputs and costs, making it possible to use the same biosensor construct in different stages of the experiment, from broad screens to individual strain characterizations.

Another very interesting possible output system is selection. The biosensor is coupled to the expression of a protein that either enables survival under certain conditions, like the expression of antibiotic resistance genes or toxin/antitoxin pairs, or they lead to cell death, as for example the expression of a lytic protein. Selection systems can achieve very high throughput rates of more than 10^9 variants per day (Dietrich *et al*, 2010) and colony formation assays can be carried without the need for additional instruments. Selection systems also offer the possibility to incorporate them in generic circuits to build self-controlling circuits.

A) Transcription factor-based biosensor



B) Riboswitch-based biosensor



C) Two-component system-based biosensor

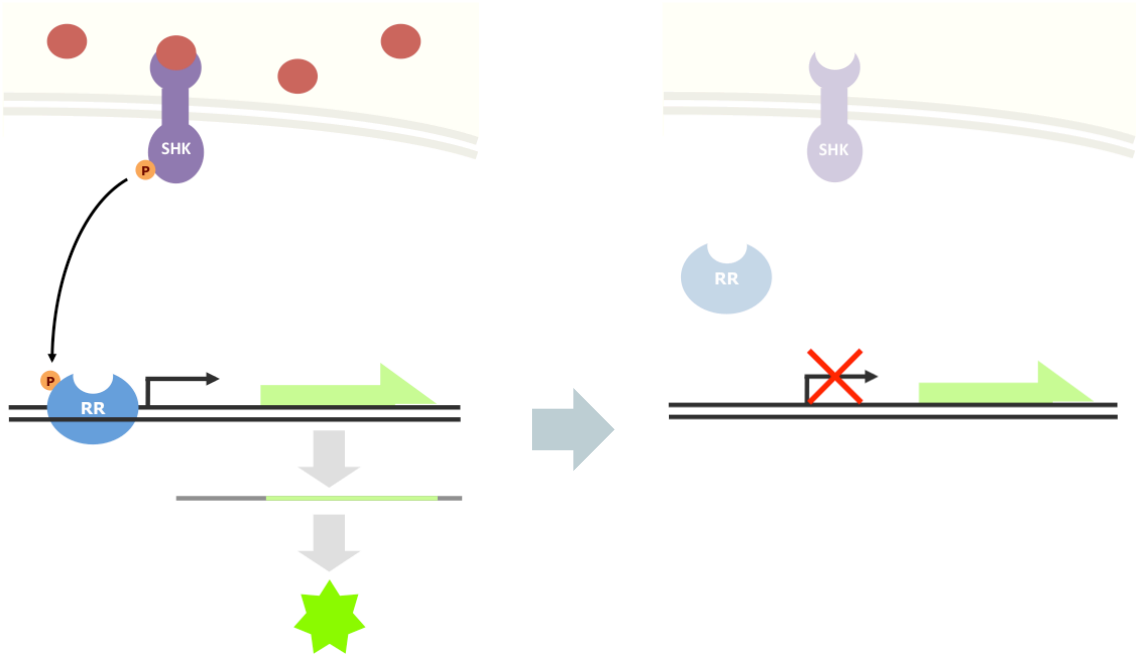


Figure 1.1) **Schematic overview of the functional mechanism of genetically encoded biosensors. (A)** Biosensor based on a repressing transcription factor (TF). In the given example is the transcription factor bound to the DNA in absence of an effector molecule (red circle). This inactivates the promoter and no reporter molecule (green star) can be transcribed and translated. Upon binding of the effector, the TF looses DNA binding affinity and releases the promoter. The gene can be transcribed and translated to the reporter molecule. **(B)** Biosensor based on a riboswitch (RS). The gene is transcribed independent of the presence of effector molecules. In the shown example is the ribosome-binding site (RBS) on the mRNA accessible in absence of effectors and thus the gene can be translated. In presence of the effector, the conformation of the mRNA changes and masks the RBS. Therefore, the mRNA can no longer be translated. **(C)** Biosensor based on a two-component system. The two-component system consists of a sensor histidine kinase (SHK), located in the outer membrane, and an intracellular response regulator (RR). The SHK is inactive in absence of an effector, but gets activated upon binding, which leads to the phosphorylation of the RR. The RR is activated by the phosphorylation and acts as an activator in this example, stimulating the expression of the reporter molecule.

1.1.2.1 Transcription factors based biosensors

Transcription factors (TFs) are regulating proteins. In microorganism, fifty-nine different classes of transcription factors are known, which all have specific structures and modes of action. The unifying characteristic is that all of them have at least a DNA-binding domain and a sensor domain. TFs change the expression rate of regulated genes upon stimulation in the sensor domain, generally by a small effector molecule (Figure 1.1A). Transcription factors can be divided in three main categories, namely repressors, activators and dual regulators. When a repressor or activator, respectively, binds to its DNA binding site in the promoter region of the controlled gene, it either represses or activates the expression. A dual regulator can have both effects.

The bacterial genome with the most thoroughly studied regulation network is *Escherichia coli*. By today, there is experimental evidence for 184 different transcription factors in the *E. coli* genome (Gama-Castro *et al*, 2016). Furthermore, there is a large number of thus far uncharacterized proteins that are classified as possible transcription factors. Current predictions anticipate that 120 additional transcription factors are part of the *E. coli* regulatory network with so far unknown stimuli and binding sites. Including the transcription factors from other microbial genomes, a total of 11,790 transcription factors are known so far (Gama-Castro *et al*, 2016).

The known TFs are responsive to at least 308 different stimuli, not including protein-protein interactions (Table S I) (Novichkov *et al*, 2013). The stimuli are often small molecules or ions, including for example amino acids, cofactors, different carbohydrates and other organic and inorganic chemicals, but there are also TFs reacting to physical changes of e.g. pH, osmolarity or temperature. Other transcription factors are activated or deactivated by protein-protein interactions or protein modifications like phosphorylation. The interaction with the effector results in a conformational change of the TF and thereby a change of the affinity of the DNA-binding domain for their DNA-binding site (Schleif, 2013). It is possible that a TF either gets activated or deactivated by these changes respectively gains or looses the ability to interact with the DNA.

A TF can have different effects on the DNA and the transcription level. The simplest effects of TFs are repression and de-repression of transcription. These two effects occur when the DNA binding site of a TF is within, overlapping or close to the promoter region of a gene or gene cluster. If the DNA affinity of the TF is increased upon stimulation, repression takes place. The binding of the active TF results in the physical blocking the promoter region and thus the

prevention of the binding of the RNA-polymerase, respectively the σ-factor guiding the RNA-polymerase. As a result, the transcription is repressed (Figure 1.1A). In the opposite case, de-repression, the DNA affinity decreases upon stimulation of the TF. The promoter gets released by the now inactive TF and is again accessible for the RNA-polymerase, which leads to an increase of transcription. The most well-known examples for a de-repressor are the *lac* and the *tet* transcription factors (LacI and TetR) (Gilbert & Müller-Hill, 1966). The opposite effect, repression, can be observed with the tryptophan repressor (Trp). The repressor is inactive and soluble until tryptophan binds. The binding of tryptophan increases the DNA affinity, Trp binds downstream of the promoter and prevents the RNA-polymerase from binding.

Repression can also take place when the binding of the TF causes conformational changes in the DNA. In the case of AraC (Lutz, 1997), the active form induces the formation of a loop with the DNA, which blocks the promoter. Interestingly, AraC is a dual regulator that can also activate transcription in its active form when arabinose is present by recruiting the RNA-polymerase. Another way of activating transcription is by bending the DNA, widening the groove and making the promoter more accessible for the RNA-polymerase. This mechanism can be observed with the Cra-activated *ppsA* promoter (Nègre *et al*, 1998).

The possible interactions and effects of TFs are even more complex, as one gene or gene cluster is typically controlled by several TFs with often opposing effects. Furthermore, TFs can also interact with each other, altering their activities and affinities. Cyclic-AMP receptor protein (CRP), formally known as catabolite activator protein (CAP), is a good example for the complexity of the transcriptional regulation network. CRP is one of the most well studied, globally acting transcription factors, controlling the activation of more than 100 genes in the *E. coli* genome in response to the intracellular cAMP concentration (Keseler *et al*, 2013). CPR is a dual regulator that either activates (e.g. *gapA*) or represses (e.g. *gdhA*) the expression and in most cases, it interacts with a number of other TF in order to fine-tune the metabolism and adjust it to the current environment (Busby & Ebright, 1999; Shimada *et al*, 2011a; Green *et al*, 2014). CRP is additionally controlling the expression of several other TF, for example AraC, adding another level of complexity. Furthermore, the native DNA-binding sites rarely resemble the DNA sequence of the highest affinity of the TF, but instead consist of slightly changed sequences (Novichkov *et al*, 2013). This is a way in which the strength of transcription control is adjusted to modulate and fine-tune the transcription levels of different genes, controlled by one TF. Another factor that can influence the efficiency of a TF is the position of its binding site on the DNA and the number of binding sites upstream of one gene or gene cluster.

As this very brief overview suggests, the transcriptional regulation network, even only regarding transcription factors, is highly complex and still far from being fully understood. The possibilities of identifying and testing new TF binding sites is steadily improving with *in vivo* (Boeva, 2016) and *in vitro* (Shimada *et al*, 2011b; Franco-Zorrilla & Solano, 2016) screens and also the reliability of *in silico* predictions increases steadily with advanced machine learning tools (Li *et al*, 2015). Nevertheless, it is thus far still impossible to reliably predict the effect or strength of an uncharacterized TF binding site.

1.1.2.2 Transforming TF to biosensors

Regardless of the obstacles of identifying the full effects and regulation networks of TFs, there are several characteristics that make TFs particularly interesting targets for the development of novel biosensors. As TFs comprise of a protein as well as a targeted DNA component, there are several options to tweak, alter and optimize TFs in order to convert them to applicable biosensors.

In certain cases, it is sufficient to choose a transcription factor with the desired sensitivity and clone a native promoter containing the respective TF binding site upstream of a reporter gene in order to generate a functional biosensor (Siedler *et al*, 2014a). Oftentimes, though, the native TF/binding-site pair has not yet the required characteristics to be used as a biosensor and requires optimization or *de novo* development, if there is no suitable native TF available.

The qualities of a biosensor are defined by its specificity to the ligand of interest, its sensitivity range for this ligand, its dynamic range of output between ON and OFF state and the general “leakiness” during the OFF state, meaning the baseline expression rate of the reporter protein. Furthermore, there are often host incompatibilities when a biosensor is transferred from one organism to another (Blazeck & Alper, 2013; Zhang *et al*, 2015; Skjoedt *et al*, 2016). Depending on the desired application, different qualities can be important.

If the biosensor should be applied in a production pathway, the sensitivity for the target usually needs to be decreased. In a product strain, the expected yield is exceeding the physiological concentrations of the respective product and therefore saturates at concentrations below the expected or desired concentrations. In the case that the biosensor controls the expression of a toxic gene, a very tight regulation with a low OFF state expression

is essential. A biosensor with a broad dynamic range is important, when a very sensitive feedback is necessary.

In the recent years, a number of functional biosensors have been published that were tuned with modern technologies of protein engineering and synthetic biology in order to overcome these problems. As an example, in a recent study by Taylor et al., they successfully applied a range of protein and DNA engineering methods in order to change the specificity of LacI to novel ligands with a similar specificity and inducability compared to the wild type LacI and its inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) (Taylor *et al*, 2016).

The next section gives a more details overview on how TF biosensors can be changes and the reasons why these adaptations are necessary to transform the TF to a biosensor.

Target specificity

A biosensor needs to have a high specificity for the desired effector molecule. In certain cases, there is no native biosensor available for a specific effector in the host strain. By applying either randomizing approaches or very targeted changes, the change in specificity of a biosensor to a new molecule could be achieved.

AraC (arabinose operon regulatory protein) is a very well studied transcription factor and has also been the basis for several biosensor developments. Native AraC is sensitive to L-arabinose, but Tang et al. have successfully applied saturation mutagenesis in order to manipulate the specificity of AraC. In an initial study in 2008, Tang et al. could generate an AraC mutant with an altered specificity for D- instead of L-arabinose (Tang *et al*, 2008). In further studies, it was demonstrated that the specificity could also be tailored towards mevalonate (Tang & Cirino, 2011), TAL (Triacetic Acid Lactone) (Tang *et al*, 2013) and ectoine (Chen *et al*, 2015). Chou and Keasling also used AraC for the development of a chimeric transcription factor. The modular construction of TFs of individual domains and the high conservation of these domains makes them perfect targets for the building of chimeras. They created a novel biosensor with sensitivity for isoprenoids and the well characterized AraC regulation by fusing the sensor domain of Idi (IPP isomerase) to the DNA binding domain of AraC (Chou *et al*, 2013). Similar chimeric transcription factors have also been successfully developed in the GalR-LacI family of transcription factors (Meinhardt *et al*, 2012). Chimeric receptors are additionally highly interesting, as they can facilitate in the creation of functional logic gates that enable input of several signals in parallel without requiring several different TF binding sites on one promoter (Shis *et al*, 2014).

Detection range

The detection range of a transcription factor or biosensor is defined here as the affinity of a biosensor to its ligand, which is classically described by the dissociation constant K_d of the sensor for the respective ligand.

Dynamic range

The dynamic range of a biosensor describes the difference in signal output between the ON and the OFF state, respectively when the biosensor is fully active and fully inactive. The optimization of the dynamic range is important, as the output strength needs to match the detection system, for example the respective detection spectrum of a flow cytometer.

TF based biosensors offer the possibility of fine-tuning their dynamic range by a number of different approaches, including changing promoter sequences, the position, amount (Ang *et al*, 2013) and sequence of the TF binding site and the RBS (Alper *et al*, 2005; Bonde *et al*, 2016; Hammer *et al*, 2006; Rogers *et al*, 2015; Zhang *et al*, 2012). Modern DNA synthesis enables quick, reliable and affordable *de novo* design of DNA fragments and novel seamless DNA assembly techniques like USER cloning (Nørholm, 2010; Cavaleiro *et al*, 2015) or Gibson assembly (Gibson, 2011) facilitate a nearly restriction free creation of DNA sequences. One example of a biosensor with a highly increased dynamic range is FadR. By switching the native FadR binding promoter with phage promoters containing two FadR binding sites and combining it with an inducible LacI promoter, the dynamic range could be increased drastically. The optimized biosensor enabled the successful selection of production mutants with an increased biofuel yield (Zhang *et al*, 2012).

Orthogonality

A common obstacle in the development of novel biosensors is the transferability between different hosts. Due to differences in for example codon usage, promoter design and expression systems, especially between bacteria and yeast, it is often difficult to transfer a functional TF or biosensor from one organism to another. Although, particularly the transfer of biosensors from bacteria to yeast is interesting for biotechnology, as yeast is a common production host, but only encodes a small number of TF by itself. Consequently, the application of biosensors in yeast is relaying to a huge extend on the possibility to orthogonal transfer biosensors (Skjoedt *et al*, 2016).

It is generally considerably easier to transfer a repressor between different hosts, as their mechanism normally involves a simple physical blockage of the polymerase-binding site.

Because of this, there have already been several examples of bacterial repressors in yeast (Wang *et al*, 2016; Teo & Chang, 2015). Only in a recent study by Skjoedt and co-workers, they were able create functional activating biosensors with bacterial origin in yeast. They chose to test several members of the LysR-type transcriptional regulators (LTTRs). Based on current knowledge of the mechanism of LTTSs and the construction of yeast promoters, they rational decided on 2 different sites in the yeast promoter, where they introduced the LTTR binding sequences. This way, they were also able to create one platform, that could be used for all tested LTTRs by simply changing the TF binding site sequence in the promoter (Skjoedt *et al*, 2016).

1.1.2.3 Biosensor applications

As already shown in Table I, biosensors have already been applied in a wide range of different applications. They have been applied to successfully rise the titers in the production of compounds like amino acids (Binder *et al*, 2012a; Mustafi *et al*, 2012), succinate (Dietrich *et al*, 2013), butanol (Dietrich *et al*, 2013) and secondary metabolites (Siedler *et al*, 2014b) to just name a few examples, by either sensing the end product itself, identify new enzymes or facilitating pathway balancing.

A particularly interesting area of biosensor applications though is the development of novel circuits. In many cases of pathway development, an intermediate can be toxic to the cell in high concentrations. In these cases, TF offer the possibility to dynamically control the pathway by synthetic feedback loops. Dhal et al. were able to construct a dynamic pathway regulation to avoid the accumulation of toxic farnesyl pyrophosphate (FPP) during the production of amorphadiene, resulting in a two-fold increase in production (Dahl *et al*, 2013). A similar approach was used to improve fatty acid synthesis in *E. coli*. An acetyl-CoA or a malonyl-CoA biosensor were applied regulates the expression levels of pathway enzymes in order to balance it, according to the current intracellular availability of acetyl-CoA or malonyl-CoA, respectively. In all cases, highly increased yields could be achieved (Xu et al, 2014; Liu et al, 2015; Zhang et al, 2012).

Dynamic pathway control is, as shown in these two examples, a very strong tool for biotechnology, as they enable the individual pathway adjustment of each single cell in a population. It helps as well to avoid the accumulation of toxic products as the expression of

enzymes before the precursor is added to the fermentation process, which could possible make external inducer molecules redundant.

1.1.2.4 Two-component systems as biosensors

Bacterial two-component systems (TCSs) are related to TFs and should be briefly mentioned. TCSs comprise of two separate proteins. The response regulator (RR) has the same mechanism of binding to promoter and activating or inhibiting transcription as TF. In contrast to TFs, TCSs do not bind or sense the effector themselves. A separate receptor unit that is located in the cell membrane senses the effector, which is often an extracellular stimulus, and transmits the information by phosphorylation of the response regulator (Figure 1.1C).

Biosensors based on TCS offer the possibility to sense for effectors outside of the cell. This could be of great advantage, for example when the bacterium shall be used as a whole-cell biosensor for sensing toxins or other effectors in the environment or for co-cultivation experiments in metabolic engineering (Meyer *et al*, 2015; Tsai *et al*, 2015; Luka *et al*, 2015). The sensor domain in the periplasm allows detecting an effector that cannot cross the membrane. The receptor proteins contain a very conserved structure in the DNA spanning domain and the phosphorylation domain. This enables the creation of chimeric receptor proteins by using different effector binding domains with only one phosphorylation domain. The phosphorylation domain will then phosphorylate the same well-characterized response regulator, but upon addition of different effector molecules. Creating a platform of chimeric receptors opens to a wide range of possible novel biosensors (Yusuf & Draheim, 2015; Nørholm *et al*, 2015).

1.1.2.5 Riboswitches

The other major native expression control system that is target of biosensor development is riboswitches. As this thesis focuses on transcription factor based biosensors, this chapter will only provide a brief overview of the mechanisms of riboswitches in eukaryotes, the possibilities to synthetically create them and successful examples of riboswitch based biosensors.

In contrast to transcription factors, riboswitches are intrinsic parts of the mRNA coding for the reporter protein (Figure 1.1B). Upon binding of an effector molecule, the mRNA changes conformation. The change of conformation either allows the ribosome-binding site (RBS) to be accessible or to be folded and therefor inaccessible. The direct and fast regulation that does not need any interaction with another molecule than the effector make riboswitches a very appealing target for biosensor development. Hence, there is only a small number of known natural riboswitches available, for example for thiamine pyrophosphate (TPP) (Muranaka *et al*, 2009) or adenosylcobalamin (Nou & Kadner, 2000).

Consequently, current research is aiming for the development of synthetic, custom-made riboswitches for any desired product. A common approach is systematic evolution of ligands by exponential enrichment (SELEX), in which a large library of RNA molecules is created to fish for aptamers with the right specificity. The effector of interest is often bound to a solid membrane or beads, enabling washing steps and thereby removing RNA sequences with lower affinities. The binding and washing cycles need to be repeated several times, which is a time consuming procedure and a drawback of this technology. Instead of starting with a library of unknown sequences, it is also possible to alter the specificity of a known aptamer by introducing mutations in the sequence. There are a few successful developments of synthetic riboswitches e.g. tetracycline (Hanson *et al*, 2003) and theophylline (Suess *et al*, 2004). Even though, the transfer of *in vitro* derived aptamers to *in vivo* functional riboswitches is challenging (Berens *et al*, 2015).

Nevertheless, riboswitches as biosensors offer a range of advantages over TF based biosensors, and *vice versa*.

1.2 Transcription factor and biosensor Cra

The catabolite repressor activator (Cra) was initially named fructose repressor (FruR), as it was first described as a repressor of the fructose operon in *Salmonella typhimurium* and *E. coli* (Geerse *et al*, 1986; Chin *et al*, 1987). Later on, it was shown that Cra has a way larger role in the bacterial metabolism in some bacteria, regulating a large number of genes involved in the central carbon metabolism and beyond (Bledig *et al*, 1996a; Saier & Ramseier, 1996; Shimada *et al*, 2005, 2011b; Njoroge *et al*, 2012). In *E. coli* and *S. typhimurium*, Cra is facilitating as the major switch between glycolysis (Embden-Meyerhof-Parnas [EMP] pathway) and gluconeogenesis (Ramseier *et al*, 1993, 1995), respectively, the metabolic and non-metabolic transcriptome (Kotte *et al*, 2010).

Interestingly, it is omnipresent and highly conserved in gram-negative bacteria (Leclerc *et al*, 1990; Vartak *et al*, 1991), even in species that do not express the EMP pathway, e.g. *Pseudomonas putida*. For those organisms, the role of Cra is yet to be discovered (Leclerc *et al*, 1990; Chavarría *et al*, 2011, 2014), but it highlights its importance and makes it a very interesting target for further studies.

1.2.1 Structure and mechanism of action

Cra belongs to the GalR-LacI family of transcription factors (Leclerc *et al*, 1990; Weickert & Adhya, 1992a; Scarabel *et al*, 1995; Penin *et al*, 1997). In its soluble form, it forms a homo-tetramer (Cortay *et al*, 1994) with a C-terminal effector binding domain and an N-terminal helix-turn-helix (HTH) DNA binding domain (Vartak *et al*, 1991; Weickert & Adhya, 1992b; Penin *et al*, 1997). It is a pleiotropic transcription factor that binds to an imperfect, 16- respectively 18-bp long palindromic sequence (Ramseier *et al*, 1993, 1995; Nègre *et al*, 1996; Shimada *et al*, 2005, 2011b).

When Cra is bound to the DNA, it can activate and repress the transcription of downstream genes. The current state of knowledge suggests that the activating and repressing effects depend on the positioning of the binding sites relatively to the transcription start site (Shimada *et al*, 2011b). They applied SELEX in order to identify new binding sites of Cra and refine the consensus sequence (Figure 1.2A), tested the response to Cra of the different binding sites and created a map of activating respectively repressing activity in dependency of the transcription initiation site (Figure 1.2B). The general trend suggests that a positioning downstream or overlapping with the promoter results in the inhibition of transcription start, whereas a positioning upstream of the promoter region tends to activate the transcription.

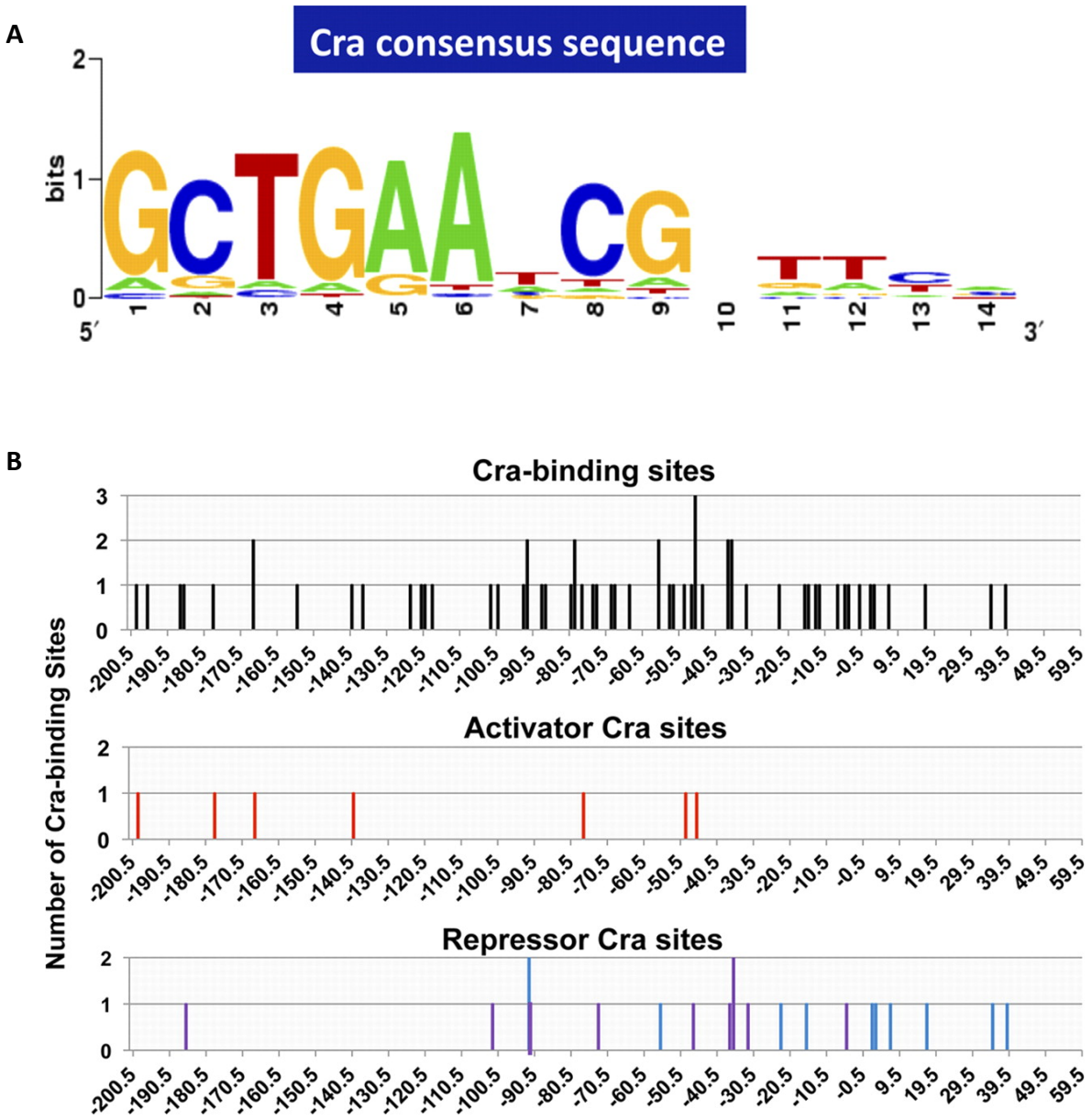


Figure 1.2) **Results of the SELEX study of the Cra binding sites by Shimada et al. (2011) (A)** Consensus sequence of the Cra binding site, computed based on the 164 binding sites that were identified by SELEX. **(B)** Positioning of the Cra binding sites of all identified promoter regions in relation to the transcription initiation site (Shimada *et al*, 2011b).

Cra is sensitive to the effectors fructose-1,6-bisphosphate (FBP) and fructose-1-phosphate (F1P) that both bind to the effector binding domain of Cra. The structural conformation of Cra when FBP or F1P are bound inhibits DNA-binding, leading to a general inactivation of Cra Figure 1.3.

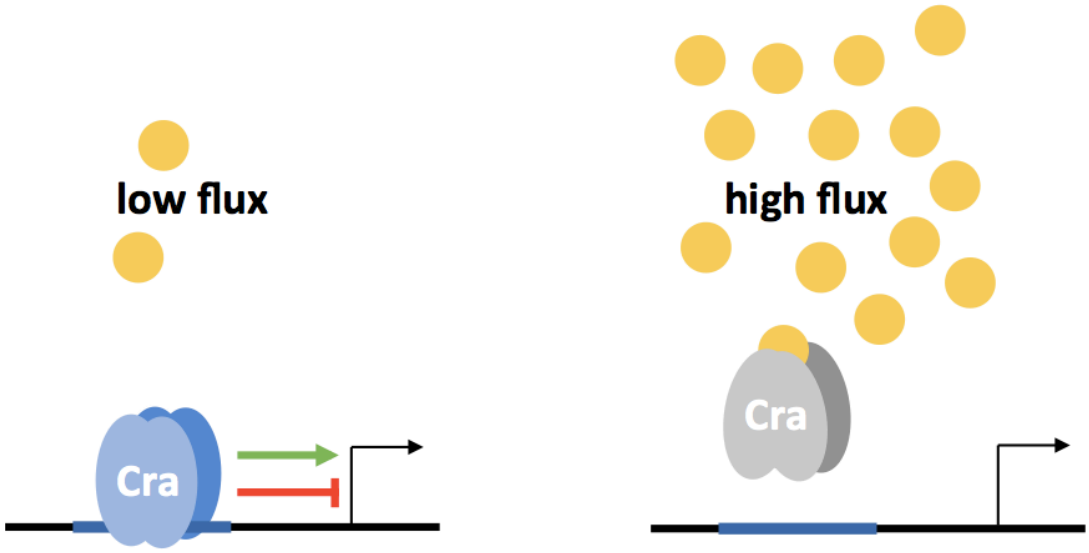


Figure 1.3) **Mechanism of the transcription factor Cra (blue: active, gray: inactive).** In a low glycolytic flux condition, the concentration of FBP (orange circles) is low and Cra active. Active Cra binds to its binding site on the DNA and can either function as an activator or repressor. In high flux conditions, the FBP concentration is high. FBP binds to Cra, which leads to a conformational change and the release of the DNA and its inactivity.

The actual global function of Cra as a glycolytic flux sensor has been, and still is, a controversial subject. One of the main reasons for this controversy is the considerably higher K_d for binding of FBP compared to F1P, which are in the mM and µM range, respectively (Ramseier *et al*, 1993). The question has been raised, why a central flux sensor would have a higher affinity to a metabolite of a branch pathway and even, whether the sensitivity for FBP might have been an artifact in the measurement.

Two recent studies on Cra in different organism support an evolutionary explanation for the differences in the K_ds. Chavarría *et al*. (2014) could show that *P. putida* is solely sensitive to F1P and does not respond to FBP, whereas Kochanowski *et al*. (2013b) could validate the FBP sensitivity of Cra in *E. coli*. Considering these two findings and the omnipresence and conservation of Cra in gram-negative bacteria, it can be hypothesized that Cra adapted to its different roles in the context of the different metabolism it faces in different organisms (Chavarría *et al*, 2011, 2014).

1.2.2 Regulon and flux dependency

The regulation network has been expanded a lot since its initial characterization as a regulator of the fructose operon (Geerse *et al*, 1986; Chin *et al*, 1987). With further research, the pleiotropic properties and Cra's importance for the regulation of carbon metabolism were -and still are- revealed.

Already in the beginning of the research on Cra in the 1990s, it became clear that it acts as a major switch between sugar catabolism and gluconeogenesis (Ramseier et al, 1993; Ramseier, 1996; Ramseier et al, 1995; Saier & Ramseier, 1996). In its active form, Cra represses the transcription of core enzymes of the glycolytic pathway, like the genes for the 6-phosphofructokinase I (pfkA) and the pyruvate kinase I (pykF). At the same time, it activates the expression of gluconeogenic genes like the phosphoenolpyruvate synthetase (ppsA) and the fructose-1,6-bisphosphatase I (fbp) (Chin et al, 1989; Ramseier, 1996). With the development of new techniques to study protein-DNA interactions, also the Cra-regulation network extended. In two SELEX studies, carried out by Shimada et al. (2005 & 2011), it could be shown that Cra is actually involved in the regulation of far more enzymes of the core metabolism, also outside of glycolysis and gluconeogenesis, as it is shown in Figure 1.4. Shimada et al. could for example show that besides repression of the genes pykA and pykF, which both encode for the pyruvate kinase, also the three genes coding for the subunits of the pyruvate dehydrogenase (PDH) complex, *aceE* (PDH decarboxylase component E1), *aceF* (PDH dihydrolipoyltransacetylase component E2) and *lpd* (lipoamide dehydrogenase, PDH component E3) are repressed. Hence, Cra does not only repress the glycolysis itself but also the further downstream energy metabolism that follows. It also represses the key operon *cyoABCDE* for the terminal electron transport system. Furthermore, Cra is also involved in the repression of the gene *zwf*, which encodes for the glucose 6-phosphate-1-dehydrogenase, the entry point to the pentose-phosphate (PP) pathway (Rowley & Wolf, 1991). Cra is furthermore involved in the regulation of certain phosphotransferase systems (PTS) that enable the transport of fructose, mannose, mannitol and to a certain extend glucose over the membrane (Zubay *et al*, 1970; Busby & Ebright, 1999; Shimada *et al*, 2011a). As recent ChiP-exo experiments in our group indicate, the regulation network of Cra is even more complex and more sensitive to changes of the metabolic condition of the cell than currently known (Arkan Vasie, unpublished data).

In a study from 2012 by Njoroge *et al.* was shown that additionally to the regulation on the DNA level, Cra also interacts with the transcriptional activator KdpE on a protein-protein level

and that Cra is relevant in the virulence in pathogenic enterohemorrhagic *Escherichia coli* (EHEC) (Njoroge *et al*, 2012).

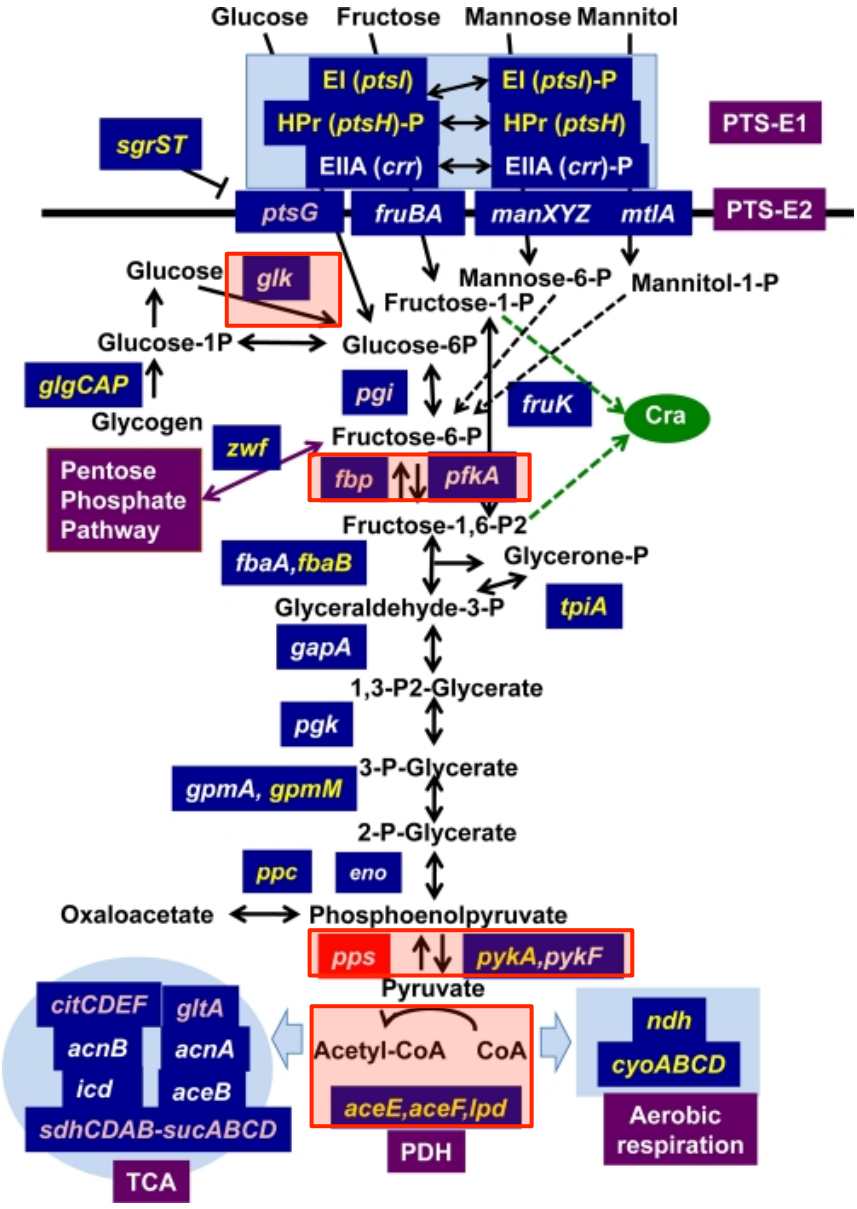


Figure 1.4) **Cra regulon described by Shimada *et al.* (2011).** The genes in blue boxes are under the control of Cra, although the genes in purple are not yet experimentally validated. Important irreversible, respectively rate-controlling, steps are highlighted by a red box. This metabolic map gives an overview of the importance of Cra in the metabolic regulation in *E. coli.* (Modified figure based on Shimada *et al*, 2011)

1.2.3 Cra as a glycolytic flux biosensor

In a recent study by Kochanowski *et al.*, they could show that Cra's function is beyond a simple metabolite sensor, but actually capable of being a metabolic flux sensor. They could show a clear, linear correlation between the FBP concentration, respectively Cra activity, and the glycolytic flux (Figure 1.5). By using a modeling approach, they could explain the linear dependency of Cra activity on the glycolytic flux by the FBP induced feedforward activation of the pyruvate kinase (Pyk). Pyk is under allosteric control of FBP and gets activated upon its binding (Valentini *et al*, 2000). The activated Pyk catalyzes the last and irreversible step in the glycolysis, the conversation from phosphoenolpyruvate (PEP) to pyruvate. Due to this feedforward activation, the correlation between the FBP concentration and the actual glycolytic flux stays linear, even when the concentration of FBP is higher than the K_m of the FBP aldolase (Kochanowski *et al*, 2013).

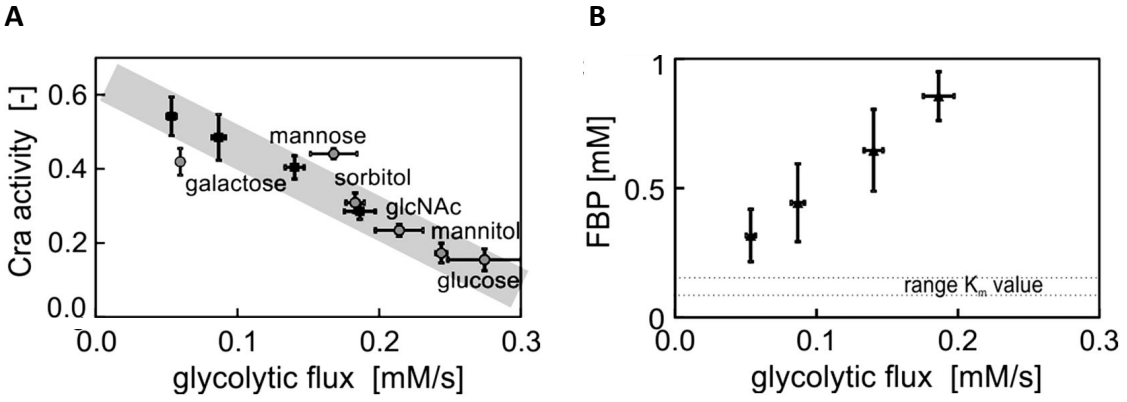


Figure 1.5) **Cra activity and fructose-1,6-bisphosphate concentration in dependency of glycolytic flux.** The study of Kochanowski *et al.* (2013) explored the correlations between glycolytic flux, Cra activity and FBP concentration. **(A)** The measured glycolytic flux, triggered by different glucose concentrations and different carbon sources, correlates with the activity of Cra. A higher glucose concentration showed as well a higher glycolytic flux as a decrease in Cra activity. The other carbon sources, which all induce different glycolytic fluxes, show the same correlation between flux and Cra activity, indicating that the effect is not substrate specific. **(B)** The intracellular FBP concentration is depending on the glycolytic flux. Figure from Kochanowski *et al.* (2013).

Furthermore, Kochanowski *et al.* developed an initial Cra-based glycolytic flux biosensor by fusing the Cra dependent promoter region of the pyruvate kinase gene (pykF) to *gfp*. In a second construct, the Cra binding site was removed from the pykF promoter region, resulting in a constitutive expression of GFP. The expression of GFP in the two different strains was monitored and used to obtain a growth-rate independent Cra-activity (Figure 1.5).

1.3 Summary

Recent technological advances as for example low cost DNA synthesis of short DNA and RNA oligonucleotides as well as full gene sequences, next generation sequencing (NGS), bacterial fluorescent activated cell sorting (FACS) and the application of robotic systems in order to generate diversity facilitate faster and more efficient developments in biotechnology. The current bottleneck, however, is the screening for interesting candidates in the abundance of variants. The promise of biosensors is that they will eventually offer cheap and customizable *in vivo* analysis tools.

An thus far unexplored area of applications is the use of biosensors in physiological studies *in vivo*. A new the current biosensor offers the possibility to reliably measure the glycolytic flux over a very broad, physiologically relevant range and convert the information into a cheap and easily detectable fluorescent output.

1.4 Aim of this project

Despite the broad range of applications of biosensors for biotechnological purposes, there has not yet been much interest in applying biosensors for studying the physiology or regulation networks in bacteria in a large-scale approach. Most biosensors are also very limited in their application spectrum, as their effector molecule limits the versatility and changing the specificity. Even though this is possible as shown in several examples, it ultimately means the development of a novel biosensor, including optimization and verification.

The aim of this PhD project is to develop a glycolytic flux biosensor suitable for high-throughput genotype to phenotype association studies. Besides verifying its applicability for possible biotechnology setups, it will be used to characterize the effect of thousands of individual *E. coli* gene knockouts on the glycolytic flux.

A biosensor of this kind would have versatile possible applications, ranging from improving the development speed for new production pathways, characterizing the metabolic state of different genotypes or build a genetic circuit in combination with product sensors in order to monitor or even control the production efficiency of each individual bacterium in a population.

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Appendix – Chapter 1

Table S I) Comprehensive list of known stimuli for transcription factors and riboswitches. Source: RegPrecise (Novichkov *et al*, 2013)

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
Amino Acids				
4-hydroxyproline	11 (37)	0 (0)	11 (11)	103
Arginine	29 (239)	0 (0)	29 (29)	249
Asparagine	1 (5)	0 (0)	1 (1)	11
Betaine	3 (11)	0 (0)	3 (3)	46
Branched-chain amino acids	3 (33)	0 (0)	3 (3)	33
Choline	1 (5)	0 (0)	1 (1)	11
cis-Urocanic acid	19 (117)	0 (0)	19 (19)	172
Glutamate	3 (15)	0 (0)	3 (3)	18
Glycine	3 (35)	18 (145)	21 (21)	188
Histidine	8 (55)	0 (0)	8 (8)	55
Homocysteine	17 (134)	0 (0)	17 (17)	171
L-citrulline	1 (8)	0 (0)	1 (1)	11
L-glutamine	0 (0)	1 (9)	1 (1)	14
LL-2,6-Diaminopimelate	1 (12)	0 (0)	1 (1)	12
Lysine	1 (4)	10 (102)	11 (11)	134
O-acetyl-L-serine	4 (43)	0 (0)	4 (4)	33
Ornithine	1 (8)	0 (0)	1 (1)	11
Phenylalanine	8 (67)	0 (0)	8 (8)	70
Proline	12 (42)	0 (0)	12 (12)	122
S-adenosylhomocysteine	10 (70)	9 (67)	19 (19)	151
S-adenosylmethionine	8 (74)	16 (130)	24 (24)	222
Tryptophan	10 (59)	0 (0)	10 (10)	83
Tyrosine	8 (67)	0 (0)	8 (8)	70
Aminoacyl-tRNAs				
Ala-tRNA	0 (0)	5 (62)	5 (5)	68
Arg-tRNA	0 (0)	3 (26)	3 (3)	46
Asn-tRNA	0 (0)	5 (43)	5 (5)	68
Asp-tRNA	0 (0)	4 (24)	4 (4)	61
Cys-tRNA	0 (0)	4 (32)	4 (4)	53
Gly-tRNA	0 (0)	7 (64)	7 (7)	78
His-tRNA	0 (0)	3 (13)	3 (3)	50
Ile-tRNA	0 (0)	9 (92)	9 (9)	95
Leu-tRNA	0 (0)	4 (53)	4 (4)	53
Lys-tRNA	0 (0)	3 (8)	3 (3)	46
Met-tRNA	0 (0)	5 (45)	5 (5)	68
Phe-tRNA	0 (0)	6 (70)	6 (6)	73

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
Pro-tRNA	0 (0)	4 (31)	4 (4)	51
Ser-tRNA	0 (0)	6 (62)	6 (6)	73
Thr-tRNA	0 (0)	6 (70)	6 (6)	73
Trp-tRNA	0 (0)	6 (66)	6 (6)	73
Tyr-tRNA	0 (0)	4 (46)	4 (4)	53
Val-tRNA	0 (0)	7 (66)	7 (7)	78
Antibiotics				
3,3'-neotrehalosadiamine	1 (3)	0 (0)	1 (1)	11
Bacillibactin	1 (5)	0 (0)	1 (1)	11
Chloramphenicol	1 (5)	0 (0)	1 (1)	12
Distamycin	1 (4)	0 (0)	1 (1)	7
Fe-Bacillibactin	1 (5)	0 (0)	1 (1)	11
Methicillin	1 (1)	0 (0)	1 (1)	7
Penicillin G	1 (1)	0 (0)	1 (1)	7
Ramoplanin	1 (7)	0 (0)	1 (1)	11
Tetracycline	1 (5)	0 (0)	1 (1)	12
Carbohydrates				
2-deoxy-5-keto-D-gluconate 6-phosphate	2 (5)	0 (0)	2 (2)	26
2-keto-3-deoxy-6-phosphogluconate	13 (95)	0 (0)	13 (13)	110
2-keto-3-deoxygluconate	6 (33)	0 (0)	6 (6)	64
2-keto-D-gluconate	4 (19)	0 (0)	4 (4)	34
5-dehydro-D-gluconate	1 (4)	0 (0)	1 (1)	12
6-phosphogluconate	1 (13)	0 (0)	1 (1)	15
Aldotetraouronic acid	1 (4)	0 (0)	1 (1)	11
Allolactose	1 (5)	0 (0)	1 (1)	12
Allose-6-phosphate	2 (3)	0 (0)	2 (2)	19
Alpha-galactosides	2 (8)	0 (0)	2 (2)	21
Alpha-glucoside	2 (12)	0 (0)	2 (2)	30
Alpha-mannans	3 (6)	0 (0)	3 (3)	11
Arabinan	1 (6)	0 (0)	1 (1)	11
Arabinogalactan oligosaccharides	1 (1)	0 (0)	1 (1)	11
Arabinose	7 (47)	0 (0)	7 (7)	84
Ascorbate-6-phosphate	7 (26)	0 (0)	7 (7)	47
Beta-galactosides	8 (29)	0 (0)	8 (8)	67
Beta-glucoside	16 (52)	0 (0)	16 (16)	103
Beta-glucoside-6-phosphate	4 (15)	0 (0)	4 (4)	34
Cellobiose	5 (22)	0 (0)	5 (5)	39
Cellobiose-6-phosphate	7 (26)	0 (0)	7 (7)	37
Chitobiose	1 (8)	0 (0)	1 (1)	11
Chondroitin sulphate	1 (7)	0 (0)	1 (1)	11
D-allose	1 (2)	0 (0)	1 (1)	12
D-galactose-6-phosphate	2 (15)	0 (0)	2 (2)	30

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
D-gluconate	2 (7)	0 (0)	2 (2)	11
D-glucono-delta-lactone	1 (3)	0 (0)	1 (1)	11
D-glycerate	8 (28)	0 (0)	8 (8)	66
D-mannonate	3 (10)	0 (0)	3 (3)	39
Deoxyribose-5-phosphate	2 (10)	0 (0)	2 (2)	26
Ethanolamine	5 (21)	0 (0)	5 (5)	59
Fructose	2 (12)	0 (0)	2 (2)	25
Fructose-1,6-bisphosphate	3 (41)	0 (0)	3 (3)	30
Fructose-1,6-diphosphate	9 (66)	0 (0)	9 (9)	75
Fructose-1-phosphate	13 (55)	0 (0)	13 (13)	125
Fructose-6-phosphate	5 (44)	0 (0)	5 (5)	56
Fucose	2 (9)	0 (0)	2 (2)	21
Galactarate	1 (2)	0 (0)	1 (1)	11
Galactobiose	1 (6)	0 (0)	1 (1)	10
Galactonate	2 (7)	0 (0)	2 (2)	24
Galactosamine-6-phosphate	7 (18)	0 (0)	7 (7)	57
Galactose	11 (61)	0 (0)	11 (11)	128
Galactose-6-phosphate	1 (4)	0 (0)	1 (1)	7
Galacturonate	7 (25)	0 (0)	7 (7)	88
Galacturonate oligosaccharides	1 (8)	0 (0)	1 (1)	11
Gluconate	19 (80)	0 (0)	19 (19)	192
Glucosamine-6-phosphate	10 (54)	6 (44)	16 (16)	147
Glucose	7 (25)	0 (0)	7 (7)	22
Glucuronate	13 (38)	0 (0)	13 (13)	132
Heparin	1 (4)	0 (0)	1 (1)	11
Hyaluronan	1 (7)	0 (0)	1 (1)	11
Hyaluronate oligosaccharide phosphate	1 (6)	0 (0)	1 (1)	15
Kojibiose	3 (9)	0 (0)	3 (3)	23
L-fuculose-1-phosphate	3 (7)	0 (0)	3 (3)	42
L-galactose	1 (6)	0 (0)	1 (1)	11
L-gulonate	3 (10)	0 (0)	3 (3)	39
L-idonate	2 (11)	0 (0)	2 (2)	27
L-talarate	3 (5)	0 (0)	3 (3)	29
L-xylulose	1 (1)	0 (0)	1 (1)	10
Lactose	2 (15)	0 (0)	2 (2)	25
Maltose	23 (126)	0 (0)	23 (23)	154
Maltose-6-phosphate	3 (10)	0 (0)	3 (3)	18
Mannitol	2 (5)	0 (0)	2 (2)	31
Mannose	8 (22)	0 (0)	8 (8)	55
Mannose-6-phosphate	1 (1)	0 (0)	1 (1)	6
N-acetylgalactosamine	3 (17)	0 (0)	3 (3)	30
N-acetylgalactosamine-6-phosphate	8 (20)	0 (0)	8 (8)	59
N-acetylglucosamine	16 (71)	0 (0)	16 (16)	134

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
N-acetylglucosamine-6-phosphate	11 (80)	0 (0)	11 (11)	106
N-acetylmannosamine-6-phosphate	3 (8)	0 (0)	3 (3)	29
N-acetylmuramate-6-phosphate	5 (18)	0 (0)	5 (5)	60
N-acetylneuraminic acid	4 (30)	0 (0)	4 (4)	46
Nigerose-6-phosphate	2 (3)	0 (0)	2 (2)	30
O-glycans	2 (2)	0 (0)	2 (2)	11
Pectic galactan	2 (9)	0 (0)	2 (2)	11
Psicose	1 (4)	0 (0)	1 (1)	15
Raffinose	1 (4)	0 (0)	1 (1)	12
Rhamnose	15 (56)	0 (0)	15 (15)	125
Rhamnulose-1-phosphate	1 (5)	0 (0)	1 (1)	11
Rhamogalacturonate oligosaccharides	3 (13)	0 (0)	3 (3)	38
rhizopine	1 (5)	0 (0)	1 (1)	15
Ribitol	1 (1)	0 (0)	1 (1)	15
Ribose	25 (121)	0 (0)	25 (25)	222
Sialic acid	1 (3)	0 (0)	1 (1)	10
Sorbitol	3 (13)	0 (0)	3 (3)	41
Sorbitol-6-phosphate	1 (5)	0 (0)	1 (1)	12
Sorbose	1 (1)	0 (0)	1 (1)	12
Sucrose	13 (57)	0 (0)	13 (13)	127
Sucrose-6-phosphate	10 (43)	0 (0)	10 (10)	90
Tagatose-6-phosphate	2 (5)	0 (0)	2 (2)	30
Trehalose	8 (36)	0 (0)	8 (8)	85
Trehalose-6-phosphate	14 (66)	0 (0)	14 (14)	115
Xylitol	3 (5)	0 (0)	3 (3)	40
Xylose	10 (57)	0 (0)	10 (10)	86
Coenzymes				
Adenosylcobalamin	4 (23)	21 (197)	25 (25)	230
Biotin	28 (222)	0 (0)	28 (28)	252
Flavin mononucleotide	8 (44)	36 (247)	44 (44)	377
Heme	3 (32)	0 (0)	3 (3)	39
NAD	1 (11)	0 (0)	1 (1)	12
NADH	18 (156)	0 (0)	18 (18)	138
Niacin	7 (57)	0 (0)	7 (7)	77
Pre-queuosine	0 (0)	2 (17)	2 (2)	30
Pre-queuosine1	0 (0)	7 (51)	7 (7)	92
Pyridoxal-5-phosphate	61 (161)	0 (0)	61 (61)	337
Tetrahydrofolate	0 (0)	3 (22)	3 (3)	50
Thiamine phosphate	4 (28)	0 (0)	4 (4)	28
Thiamine pyrophosphate	0 (0)	40 (365)	40 (40)	380
Heterocyclic Compounds				
Bis-indoles	1 (4)	0 (0)	1 (1)	7
Catechin	1 (2)	0 (0)	1 (1)	11

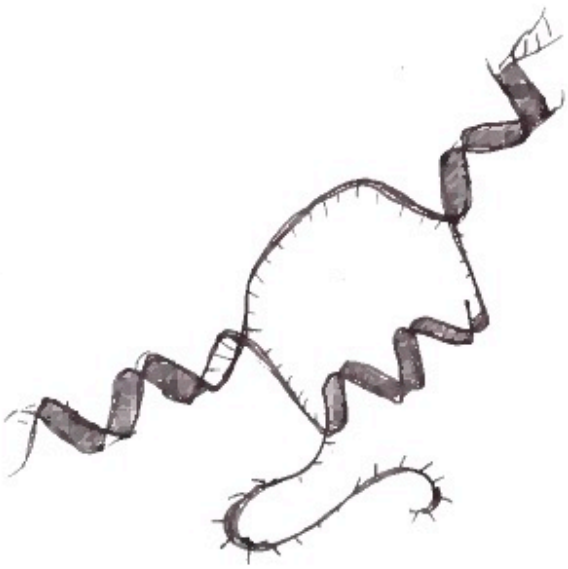
Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
Fisetin	2 (6)	0 (0)	2 (2)	11
Flavonoids	1 (2)	0 (0)	1 (1)	11
Paraquat	18 (85)	0 (0)	18 (18)	164
Proflavin	1 (12)	0 (0)	1 (1)	12
Quercetin	2 (6)	0 (0)	2 (2)	11
Rhodamine 6G	1 (12)	0 (0)	1 (1)	12
Inorganic Chemicals				
Arsenate	5 (30)	0 (0)	5 (5)	38
Arsenite	7 (45)	0 (0)	7 (7)	53
Cadmium, ion (Cd2+)	25 (156)	0 (0)	25 (25)	215
Cobalt ion, (Co2+)	5 (36)	0 (0)	5 (5)	49
Copper ion, (Cu+)	27 (156)	0 (0)	27 (27)	228
Copper ion, (Cu2+)	6 (62)	0 (0)	6 (6)	67
Hydrogen peroxide	7 (67)	0 (0)	7 (7)	67
Inorganic phosphate	3 (32)	0 (0)	3 (3)	35
Iron ion, (Fe2+)	35 (304)	0 (0)	35 (35)	268
Lead ion, (Pb2+)	18 (96)	0 (0)	18 (18)	170
Magnesium ion, (Mg2+)	0 (0)	9 (44)	9 (9)	100
Manganese ion, (Mn2+)	24 (173)	0 (0)	24 (24)	171
Mercury ion, (Hg2+)	13 (32)	0 (0)	13 (13)	129
Molybdate	29 (126)	0 (0)	29 (29)	221
Molybdenum	1 (7)	16 (81)	17 (17)	175
Nickel ion, (Ni2+)	20 (88)	0 (0)	20 (20)	193
Nitrate	5 (46)	0 (0)	5 (5)	53
Nitric oxide	45 (240)	0 (0)	45 (45)	254
Nitrite	8 (70)	0 (0)	8 (8)	88
Oxygen	13 (115)	0 (0)	13 (13)	119
Potassium ion	2 (27)	0 (0)	2 (2)	28
Silver ion, (Ag+)	5 (37)	0 (0)	5 (5)	37
Sulfate	5 (20)	0 (0)	5 (5)	42
Sulfite	2 (10)	0 (0)	2 (2)	19
Tetrathionate	4 (14)	0 (0)	4 (4)	47
Thiosulfate	4 (37)	0 (0)	4 (4)	37
Tungsten	4 (23)	0 (0)	4 (4)	30
Zinc ion, (Zn2+)	60 (446)	0 (0)	60 (60)	362
Lipids and Fatty Acids				
Gamma-linolenic acid	2 (8)	0 (0)	2 (2)	26
Linoleate	2 (8)	0 (0)	2 (2)	26
Long-chain acyl-ACP	2 (24)	0 (0)	2 (2)	30
Oleate	14 (84)	0 (0)	14 (14)	136
Oleoyl-CoA	7 (71)	0 (0)	7 (7)	73
Palmitoyl-CoA	7 (71)	0 (0)	7 (7)	73
Phytanate	2 (8)	0 (0)	2 (2)	26

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
Unsaturated acyl-ACP	12 (78)	0 (0)	12 (12)	90
Unsaturated acyl-CoA	12 (78)	0 (0)	12 (12)	90
Nucleotides and Nucleosides				
5-phosphoribosyl 1-pyrophosphate	4 (48)	0 (0)	4 (4)	48
Adenine	0 (0)	8 (69)	8 (8)	105
Adenosine	1 (4)	0 (0)	1 (1)	15
Adenosine diphosphate ribose	26 (97)	0 (0)	26 (26)	263
Cyclic 3',5'-AMP	4 (49)	0 (0)	4 (4)	50
Cyclic diguanylate	0 (0)	9 (50)	9 (9)	104
Deoxyinosine	1 (4)	0 (0)	1 (1)	12
Deoxyribonucleotides	43 (363)	0 (0)	43 (43)	378
Guanine	3 (31)	8 (69)	11 (11)	126
Guanosine 3',5'-bis(diphosphate)	1 (10)	0 (0)	1 (1)	12
Guanosine triphosphate	2 (26)	0 (0)	2 (2)	26
Hypoxanthine	3 (31)	0 (0)	3 (3)	31
Pyrimidine nucleoside	2 (6)	0 (0)	2 (2)	6
Uracil	19 (95)	0 (0)	19 (19)	118
Xanthine	1 (3)	0 (0)	1 (1)	6
Xanthosine	1 (4)	0 (0)	1 (1)	12
Organic Chemicals				
2,4-Dinitrophenol	1 (12)	0 (0)	1 (1)	12
2-Acetolactate	2 (28)	0 (0)	2 (2)	28
2-aminoethylphosphonate	6 (14)	0 (0)	6 (6)	43
2-methylcitrate	10 (60)	0 (0)	10 (10)	88
2-oxoglutarate	4 (29)	0 (0)	4 (4)	32
3-hydroxybenzoate	1 (2)	0 (0)	1 (1)	8
4,5-dihydroxypentan-2,3-dione	1 (6)	0 (0)	1 (1)	12
4-hydroxyphenylpyruvate	2 (17)	0 (0)	2 (2)	24
Acetate	1 (4)	0 (0)	1 (1)	11
Acetohydroxybutyrate	1 (16)	0 (0)	1 (1)	16
Acetoin	1 (6)	0 (0)	1 (1)	11
Allantoin	1 (2)	0 (0)	1 (1)	12
Carbonyl cyanide m-chlorophenylhydrazone	1 (12)	0 (0)	1 (1)	12
Choline	1 (7)	0 (0)	1 (1)	12
Citrate	7 (41)	0 (0)	7 (7)	41
Cumene hydroperoxide	1 (8)	0 (0)	1 (1)	11
Deoxyfructosyl glutamine	1 (3)	0 (0)	1 (1)	15
Diamide	13 (42)	0 (0)	13 (13)	119
Ethidium bromide	1 (12)	0 (0)	1 (1)	12
Ferulic acid	4 (12)	0 (0)	4 (4)	49
Formaldehyde	2 (14)	0 (0)	2 (2)	26
Formate	7 (29)	0 (0)	7 (7)	64

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
Fructoselysine 6-phosphate	1 (2)	0 (0)	1 (1)	11
Galangin	1 (4)	0 (0)	1 (1)	11
Gamma-aminobutyric acid	12 (39)	0 (0)	12 (12)	88
Gentisate	1 (2)	0 (0)	1 (1)	8
Glycerate	2 (20)	0 (0)	2 (2)	28
Glycerol 3-phosphate	2 (16)	0 (0)	2 (2)	22
Glycolate	8 (25)	0 (0)	8 (8)	85
Glyoxylate	1 (10)	0 (0)	1 (1)	12
Homogentisate	1 (5)	0 (0)	1 (1)	8
L-lactate	22 (100)	0 (0)	22 (22)	210
L-malate	2 (17)	0 (0)	2 (2)	30
Lactate	1 (6)	0 (0)	1 (1)	10
Malonyl-CoA	2 (18)	0 (0)	2 (2)	18
Mannopine	1 (3)	0 (0)	1 (1)	15
Methylglyoxal	1 (8)	0 (0)	1 (1)	15
p-aminobenzoyl-glutamate	1 (4)	0 (0)	1 (1)	12
p-Coumaric acid	3 (10)	0 (0)	3 (3)	41
Phenylacetyl-CoA	17 (80)	0 (0)	17 (17)	138
Protocatechuate	1 (2)	0 (0)	1 (1)	8
Putrescine	1 (9)	0 (0)	1 (1)	11
Pyruvate	7 (65)	0 (0)	7 (7)	65
Quinate	1 (2)	0 (0)	1 (1)	8
Quinone	1 (9)	0 (0)	1 (1)	11
Salicylate	2 (8)	0 (0)	2 (2)	23
Shikimate	1 (2)	0 (0)	1 (1)	8
Tamarixetin	1 (4)	0 (0)	1 (1)	11
Taurine	9 (26)	0 (0)	9 (9)	89
tert-Butyl hydroperoxide	1 (8)	0 (0)	1 (1)	11
Vanillate	10 (28)	0 (0)	10 (10)	83
Other factors				
Blue light	4 (21)	0 (0)	4 (4)	45
DNA damage	41 (327)	0 (0)	41 (41)	339
Heat shock	45 (374)	0 (0)	45 (45)	339
Iron-sulfur cluster redox state	26 (216)	0 (0)	26 (26)	223
New effector	4 (11)	0 (0)	4 (4)	40
Peptides, and Proteins				
BceS, sensor histidine kinase (bacitracin)	1 (7)	0 (0)	1 (1)	11
BlaR, sensor histidine kinase (beta-lactam antibiotics)	1 (4)	0 (0)	1 (1)	7
CelB, cellobiose-specific PTS component EIIB	2 (10)	0 (0)	2 (2)	30
ComP, sensor histidine kinase (ComX)	1 (4)	0 (0)	1 (1)	11
CssS, sensor histidine kinase	1 (5)	0 (0)	1 (1)	11

Effector	TF regulogs (regulons)	RNA regulogs (regulons)	Total regulogs (regulons)	Genomes
CysK, cysteine synthetase	2 (18)	0 (0)	2 (2)	18
Feedback-inhibited GlnA, glutamine synthetase	3 (28)	0 (0)	3 (3)	18
GlnK, sensor histidine kinase (glutamine)	1 (6)	0 (0)	1 (1)	11
GlnK-AMP, signal transduction protein	5 (16)	0 (0)	5 (5)	39
GutB, sorbitol-specific PTS EIIB component	2 (7)	0 (0)	2 (2)	30
HPr, phosphocarrier protein	19 (131)	0 (0)	19 (19)	59
HssS, sensor histidine kinase (heme)	1 (7)	0 (0)	1 (1)	7
KdpS, sensor histidine kinase	1 (2)	0 (0)	1 (1)	10
LiaS, sensor histidine kinase (bacitracin, nisin, ramoplanin, vancomycin)	1 (11)	0 (0)	1 (1)	11
LicB, lichenan-specific enzyme IIB PTS component	1 (5)	0 (0)	1 (1)	11
MalK, sensor histidine kinase (malate)	1 (5)	0 (0)	1 (1)	11
Malonyl-ACP	1 (7)	0 (0)	1 (1)	7
ManP, mannose-specific enzyme IIBCA PTS component	2 (7)	0 (0)	2 (2)	18
MtlA, mannitol-specific enzyme IICB PTS component	4 (24)	0 (0)	4 (4)	48
NreB, sensor histidine kinase (Nitrate, Nitrite)	1 (6)	0 (0)	1 (1)	7
NrgA, ammonium uptake protein	1 (10)	0 (0)	1 (1)	11
NrgB, ammonium uptake protein	1 (10)	0 (0)	1 (1)	11
Phosphorylated NtrB, signal histidine kinase	21 (193)	0 (0)	21 (21)	207
PspA regulatory protein	1 (5)	0 (0)	1 (1)	10
PyrR	0 (0)	9 (79)	9 (9)	95
RocG, glutamate dehydrogenase	2 (9)	0 (0)	2 (2)	11
SaeS, sensor histidine kinase	1 (4)	0 (0)	1 (1)	7
SdpI, signal transduction protein	1 (8)	0 (0)	1 (1)	11
SgaB, ascorbate-specific PTS system EIIB component	1 (11)	0 (0)	1 (1)	15
SgaB2, ascorbate-specific PTS system, EIIB component	1 (8)	0 (0)	1 (1)	15
SinI, antirepressor protein	1 (7)	0 (0)	1 (1)	11
Trx, thioredoxin protein	1 (12)	0 (0)	1 (1)	14
YvcQ, sensor histidine kinase	1 (4)	0 (0)	1 (1)	11
YvfT, sensor histidine kinase	1 (4)	0 (0)	1 (1)	11

CHAPTER 2 BIOSENSOR DEVELOPMENT



2.1 Abstract

Measuring, understanding and influencing the metabolic flux of bacterial cells are crucial for the development and optimization of production pathways. A metabolic flux sensor can help to understand bacterial physiology as well as refining ***in silico*** metabolic modelling. This chapter presents a novel, improved Cra-based biosensor in ***E. coli*** with the characteristics required for high-throughput screenings in flow cytometry. In order to improve the qualities of the original Cra biosensor by Kochanowski et al., different native promoters were tested to respond to different glycolytic fluxes. The best promoter of *ppsA* was chosen to control ***gfp*** expression and a mutated version without the repressor binding site controlling *rfp* expression. This allows constitutive expression of *rfp* and enables to correct for possible effects on protein expression caused by different growth phases and doubling times. The glycolytic flux dependency of the biosensor was validated for the range of physiologically relevant glycolytic fluxes in a series of different carbon sources. Additionally, the sensitivity of this Cra-biosensor was tested during the expression of mevalonate from the pMevT production pathway, developed by Martin *et al*, 2003. It could be demonstrated that the dynamic range of this biosensor exceeds the native range and therefore can also be applied in experimental setups that aim for increasing the glycolytic flux.

2.2 Introduction

This chapter focuses on creating to use the already excising Cra based glycolytic flux biosensor (Kochanowski *et al*, 2013) and develop it further. The current sensor is based on the Cra controlled *pykF* promoter region of the *E. coli* genome and was shown to give a reliable, flux-depended GFP expression in steady state measurements (Kochanowski *et al*, 2013). The aim of an improved biosensor is to be able to obtain a reliable, single cell signal output while measuring only once, at one time point during exponential growth. These characteristics are essential for the use of the biosensor in large-scale screenings. Such large scales screenings are needed to overcome bottlenecks in the identification of mutants with improved glycolytic flux, which could be redirected towards production pathways. Furthermore, the ability to quickly generate large datasets is also interesting for any kind of global network studies. A detailed introduction including successful examples of applications of biosensors in biotechnology and ways to improve them can be found in Chapter 1.1.

2.3 Materials and methods

2.3.1 Strains, plasmids and DNA-primer

E. coli Top10 and *E. coli* DH5a were used for the construction of pFlux. *E. coli* BW25113 and derived knockout strains from the KEIO collection (Baba *et al*, 2006) were used for the further characterization and application of pFlux. For clarifications, the Cra knockout strain *E. coli* BW25113 JW0078-1 is called Δ*cra* throughout this manuscript, even though it is originally labeled Δ*fruR* in the KEIO collection due to old terminologies.

Primer were ordered from Integrated DNA Technologies and pMevT was constructed by Martin *et al*, 2003.

Strains, plasmids and primers are listed in Table SII and
Table SIII.

2.3.2 Cultivation and growth media

The growth media used in this study are Luria-Bertani (LB) complex medium, Super Optimal broth with Catabolite repression (SOC) medium and M9 minimal medium (Kochanowski *et al*, 2013b) supplemented with filter-sterile trace element solution, resulting in a final concentration of 6.3 µM $ZnSO_4$, 7.0 µM $CuCl_2$, 7.1 µM $MnSo_4$, 7.6 µM $CaCl^2$ and 60 µM $FeCl_3$). The M9 medium contained 5 g/L of the respectively indicated carbon sources (fructose, glucose, mannitol, sorbitol, galactose, glycerin, sodium pyruvate or sodium acetate). When solid medium was required, the bacteria were grown on LB-agar plates. Antibiotics were added when required at the concentrations 25 µg/mL spectinomycin.

If not stated otherwise, the cells were cultured in 5 mL medium in 15 mL cultivation tubes or 2 mL medium in 24-deep well plates. The cultivation tubes were incubated at 37°C, 190 rpm in a common shaking incubator and the 24-deep well plates at 37°C and 900 rpm on a tabletop plate shaker (Titramax 1000 incubator, Heidolph Instruments GmbH, Germany).

Strains were stored in 15 % v/v glycerol at -80°C.

2.3.3 Plasmid construction

All plasmids were assembled by USER cloning (Nour-Eldin *et al*, 2006). Phusion™ U Hot Start DNA Polymerase polymerase (Thermo Fisher Scientific) or in house synthesized PfuX polymerase (Nørholm, 2010) were used for PCR amplifications with a standard thermocycler

program, matching the T_m of the respective primers. Amplified PCR products were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG), digested with DpnI FastDigest (Thermo Fisher Scientific) and the plasmid ligated with USER enzyme mix (New England Biolabs) according to the protocols. The resulting plasmids were transformed in the respective chemically competent *E. coli* strain.

The template for the pFlux backbones is pZA11MCS, which is a modular constructed plasmid backbone from EXPRESSYS with a p15A origin, ampicillin resistance, a tetracycline inducible promoter ($P_{LtetO-1}$) and multiple cloning site (MCS) (Lutz, 1997). The promoter regions were amplified directly from the *E. coli* BW25113 genome. The *gfp* sequence derives from Jensen et *al.* (unpublished data) and the *rfp* gene was obtained from the Standard European Vector Architecture database (Silva-Rocha *et al*, 2013). As a spectomycin resistance was desired and the respective EXPRESSYS was not available, the resistance was amplified from another EXPRESSYS plasmid using the primer PC055/PC060 for the spectomycin resistance and PC055/PC070 on pZA11MCS for the backbone creating the pZA41MCS plasmid. For the assembly of the plasmids pGFPppsA, pGFPppc and pGFPpykF, pZA14MCS was amplified with the primers PC004/PC031, eliminating the promoter region but maintaining the p15A origin of replication and the spectinomycin resistance marker. The different natural promoter regions were obtained by colony PCR from *E. coli* BW25113, using the primer pairs PC001/PC002, PC003/PC004 and PC005/PC006 and *gfp* with the primers PC019/PC021.

In order to generate the constitutive *ppsA* promoter with a scrambled Cra binding site, pGFPppsA was amplified with the primer pair PC063/PC064. This constitutive promoter was subsequently amplified with PC033/PC065, rfp with PC069/PC070. The pGFPppsA backbone was amplified with PC071/PC072, resulting in an opening of the backbone downstream of the *gfp* gene. The PCR products were ligated and transformed as described. The obtained plasmid is pFlux.

A list of the plasmids, primer and promoter sequences and a plasmid map of pFlux are provided in Appendix – Chapter 2.

2.3.4 Flow cytometry

To measure the fluorescence signals of the pFlux plasmid, the different *E. coli* strains were initially grown over night in LB medium at 37°C and 190 rpm. Minimal medium with 5 g/l of the respective carbon source (fructose, glucose, mannitol, sorbitol, mannose, galactose, malate, glycerin, sodium pyruvate or sodium acetate) was inoculated 1:50 with the LB pre-culture. The

cultures in minimal medium were incubated over night at 37°C and 190 rpm and subsequently used to inoculate fresh minimal medium (1:200) and grown under the same conditions for four hours. The cells were diluted in FACSFlow (BD) in order to prepare them for screening on the FACSAria (BD), equipped with a 488 nm and 561 nm LASER. To collect the GFP and RFP signals, the FITC (530/30) and PE-Texas Red (610/20) filter were used.

In order to maintain comparability between several runs on different days, the *E. coli* strain BW25113 was aligned with the diagonal between the FITC and PE-Texas Red-channel. The obtained data was analyzed with FlowJo (FlowJo LLC, Oregon, US).

2.3.5 Validation in a mevalonate production strain

The *E. coli* BW25113 strain containing the pFlux plasmid was subsequently transformed with pMevT (Martin *et al*, 2003) or pZA1 and grown in medium containing 25 ng/mL spectinomycin and 25ng/mL chloramphenicol. For mevalonate production, the cells were grown in M9 medium containing 5 g/L glucose. When transferring the cultures from the pre-culture to fresh medium, they were induced with 0.05 mM IPTG. Fluorescence was determined 5 hours after induction by flow cytometry.

2.4 Results and Discussion

2.4.1 Identification of flux dependent promoters regions

To generate a flux dependent biosensor that can be applied in high-throughput screening approaches, we tested three different promoter regions (*pykF*, *ppsA* and *ppc*). All three promoters are thus far known to be regulated exclusively by Cra (Keseler *et al*, 2013). Promoter regions are often controlled by a number of different activators and repressors that give feedback of a variety of intra- and extracellular signals (Keseler *et al*, 2013). By choosing promoter regions that are regulated by only one transcription factor, potential bias through cross-interactions with other effectors and regulators is reduced.

We tested one promoter region that gets activated (*ppsA*) by Cra and two that get repressed (*pykF*, *ppc*) (Table SIV). The promoters were cloned in front of *gfp* to enable a Cra dependent regulation of GFP output fluorescence (Figure 2.1A) The fluorescent signal was measured at the single cell level during exponential growth phase in various carbon sources using flow cytometry. The uptake of the used carbon sources as well as their entry point in the glycolysis differ and these differences generate distinct fluxes for the FBP conversion, depending on the carbon source the bacterium is utilizing.

The expression levels of the two repressing promoter regions turned out to be not suitable for the high-throughput biosensor approach. The *pykF* promoter showed a too low expression level for reliable discrimination in a high-throughput approach and the changes in expression between high and low flux state was too small in the case of the *ppc* promoter (Figure 2.1B). In the case of *ppsA* the promoter is activated under low flux conditions. After correcting for the background signal and differences in OD_{600}, a 16-fold induction of the fluorescent signal was detected after growth on acetate (15318 ± 168 a.u.) compared to glucose (830 ± 103 a.u.). When *E. coli* is grown on glucose, the expression of GFP is furthermore very low, indicating the low leakiness of the promoter in absence of active Cra molecules. The *ppsA* promoter region showed the best dynamic range as well as the tightest regulation and was chosen for further optimizations.

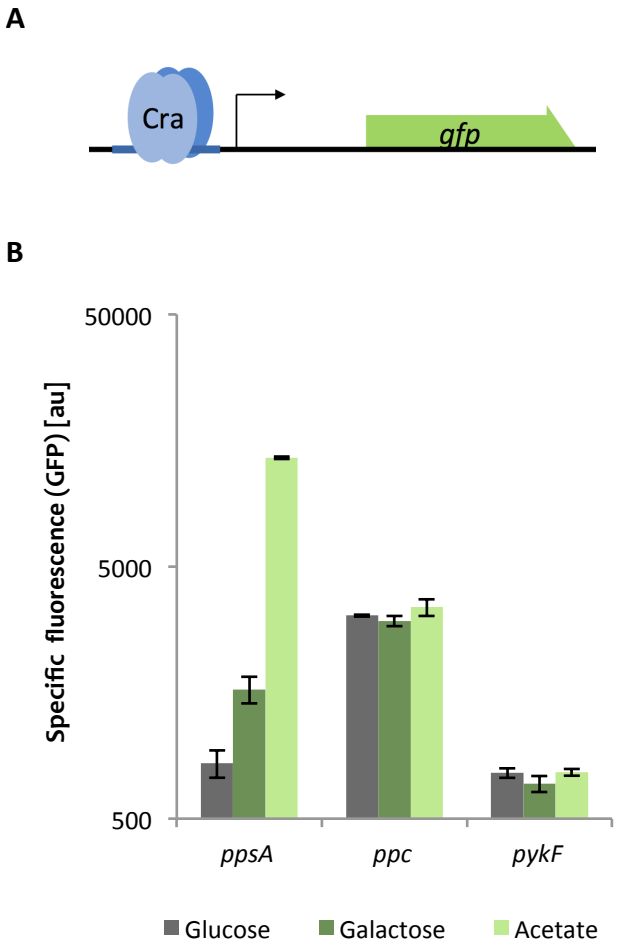


Figure 2.1 **Construction of the initial screening approach (A)** Different Cra regulated promoter regions (*ppsA*, *ppc* and *pykF*) are cloned in front of *gfp*, generating three different reporter plasmids **(B)** The fluorescence of *E. coli* KEIO wild type strain BW25113 with the different flux sensor constructs was measured by flow cytometry. The cells grew in minimal media containing carbon sources inducing high glycolytic flux (glucose/gray), medium flux (galactose/dark green) and low flux (acetate, light green). By applying a gate in the FSC/SSC dot plot, the bacterial cells could be separated from background noise. The bars represent the mean fluorescent signal of the bacteria population in the FITC channel. The standard deviations were calculated from three individual experiments.

2.4.2 Design and characterization of a robust biosensor

A robust biosensor needs to provide a reliable output even in changing conditions. This is of especial importance in the case of a flux biosensor, as the use of different carbon sources does not only lead to changes in the glycolytic flux but also in the growth rate. The growth rate, subsequently, has a not negligible influence on protein maturation rate, which can give a bias in the GFP per cell density ratio. In order to generate a biosensor with reduced noise, a two-color approach was implemented (Kosuri *et al*, 2013). The final biosensor construct contains the native *ppsA* promoter with a Cra binding site as well as a second promoter with a variant of the *ppsA* promoter with the Cra binding site sequence scrambled. The native *ppsA* promoter regulates the expression of GFP whereas the altered *ppsA* promoter with the scrambled Cra binding site controls the expression of a red fluorescent protein (RFP) (Figure 2.2A, Table SV). The expression levels of both, GFP and RFP, are equally affected by the protein maturation bias during the growth on different carbon sources. As Cra controls only the promoter upstream of *gfp*, the information about the glycolytic flux is solely conveyed into the intensity of the GFP signal, but not the RFP signal. Therefore, relative glycolytic flux can be obtained by calculating the ratio of the fluorescence intensities of GFP and RFP.

In order to conform the assumption that this construct is actually capable of eliminating possible growth defects but also show a Cra depended expression pattern, the construct was tested in a wild type *E. coli* W25113 strain and an *E. coli* W25113 Δ*cra* deletion strain. The two strains were grown in M9 medium with either glucose (dark gray) or pyruvate (light gray) as the sole carbon source and samples measured during exponential growth. Figure 2.2B shows the dot plots of the Δ*cra* strain. The overlay of the cells grown in presence of glucose (dark gray) and pyruvate (light gray) is clearly visible in this plot. The growth of the wild-type strain in the same conditions results in a distinct shift of the two populations towards a higher signal in the green channel. However, the fluorescent intensity of the bacteria grown on pyruvate (light gray) is a magnitude higher than on glucose (dark gray), resulting in two easily distinguishable populations in the dot plot (Figure 2.2C).

In order to test if the Cra biosensor is also sensitive enough to give a feedback on a variety of different fluxes in between the highest (glucose) and lowest (pyruvate) physiologically relevant fluxes, a range of other carbon sources was tested. These carbon sources have various entry points into the glycolysis and also are taken up and converted in different rates, consequently resulting in different glycolytic fluxes. The GFP/RFP ratios follow the trend that is to be expected based on the previously measured and estimated glycolytic fluxes (Figure 2.3).

Furthermore, the flux sensor provides a large dynamic range with a ratio of 2.49 ± 0.28 GFP/RFP fluorescence on fructose and 17.83 ± 0.59 on acetate. The expression level in the Δ*cra* strain on different carbon sources is not following the trend towards a higher GFP/RFP signal with lower fluxes as observed in the wild-type strain as it was to be expected. There is even a small decrease noticeable, caused by a slight shift towards a higher red signal in the strains grown on carbon sources with a low glycolytic flux.

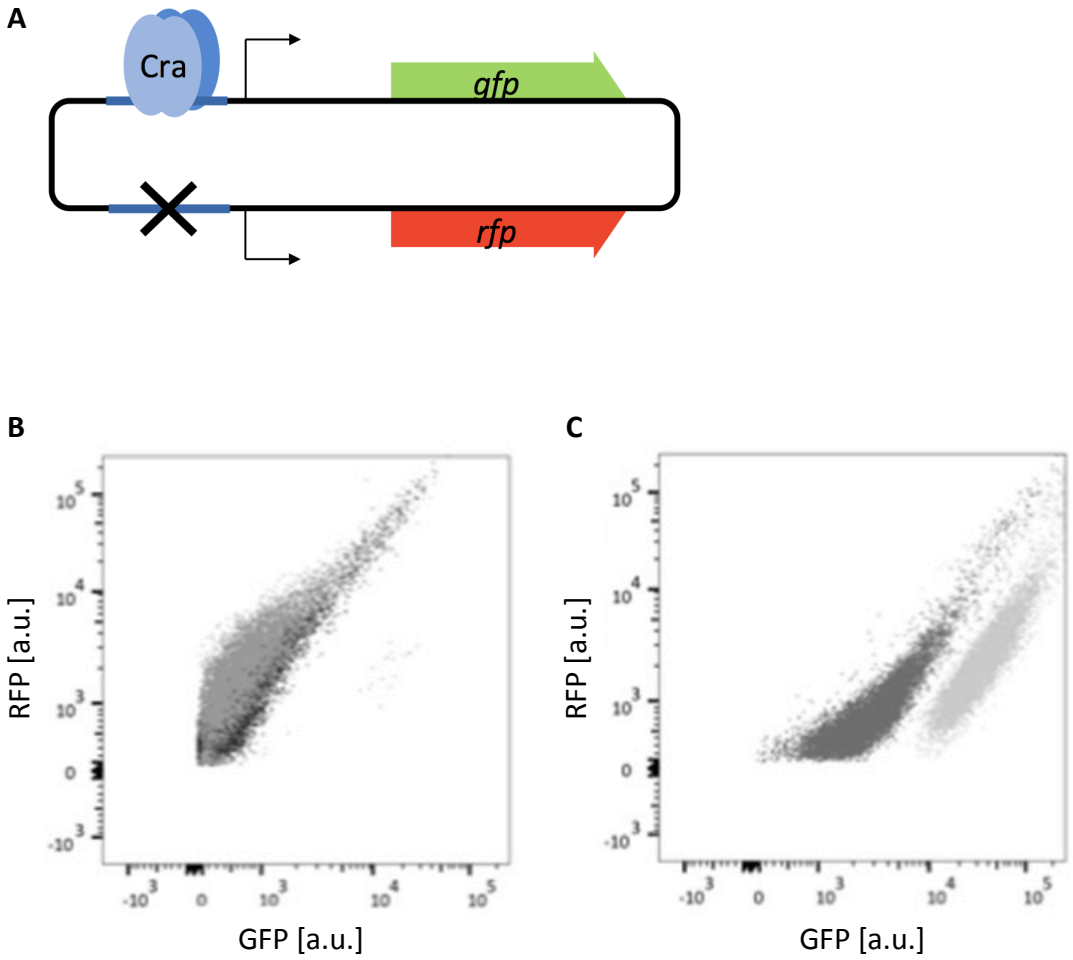


Figure 2.2) **Construct and evaluation of pFlux. (A)** Schematic map of pFlux. The transcription of *gfp* is controlled by a Cra dependent *ppsA* promoter, whereas the transcription of *rfp* is controlled by a *ppsA* promoter with a scrambled Cra binding site. **(B)** Dot plot of the GFP and RFP expression of the *E. coli* Δ*cra* strain during growth on glucose (dark gray) and pyruvate (light gray). **(C)** Dot plot of the GFP and RFP expression of *E. coli* BW25113 wild type during growth on glucose (dark blue) and pyruvate (light blue). The fluorescence signals of GFP and RFP were measured by flow cytometry for 50,000 cells per run.

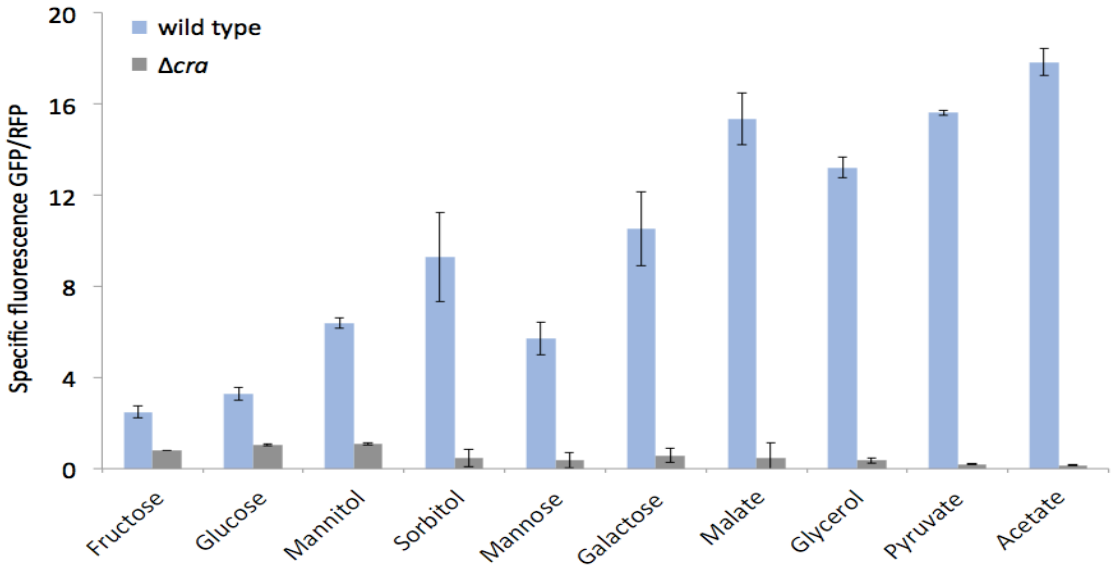


Figure 2.3) **GFP/RFP emission ratios for *E. coli* W25113 wild type (blue bars) and Δ*cra* (gray bars) when grown on different carbon sources, inducing different glycolytic fluxes.** The fluorescent signals of GFP and RFP where obtained with flow cytometry. The signal of the Δ*cra* strain grown on glucose is used as a reference point in the measurements and was positioned on the diagonal between the green and red channel. The mean value of the green (GFP) and red (RFP) channels was obtained and the ratio calculated. The average and standard deviation of three individual experiments are shown.

2.4.3 Validation of pFlux in different *E. coli* strains

In order to validate the versatility of pFlux, it was transformed into different *E. coli* strains. The selected six *E. coli* strains are common laboratory strains and comprise K- as well as 1 B-strain (BL21) in order to test the biosensor in a variety of genetic backgrounds. The strains were grown in presence of glycolytic and gluconeogenic carbon sources and the GFP and RFP signals measured by flow cytometry. As shown in Figure 2.4, all *E. coli* strains follow the same pattern as previously observed for *E. coli* BW25113. *E. coli* Crooks showed a generally higher GFP/RFP ratio which correlates to the generally lower fluorescent signals in the GFP as well as the RFP channel compared to the other *E. coli* strains, causing a numerical bias. Even though the actual expression levels and subsequent ratios slightly differ in all strains, this should not have an impact on the usability of pFlux, as the trends in flux response were comparable in all tested *E. coli* strains.

There was no growth of BL21 in M9 medium containing galactose as sole carbon source, as it contains the *gal* mutation in the galactose metabolism, making it galactose non-utilizing.

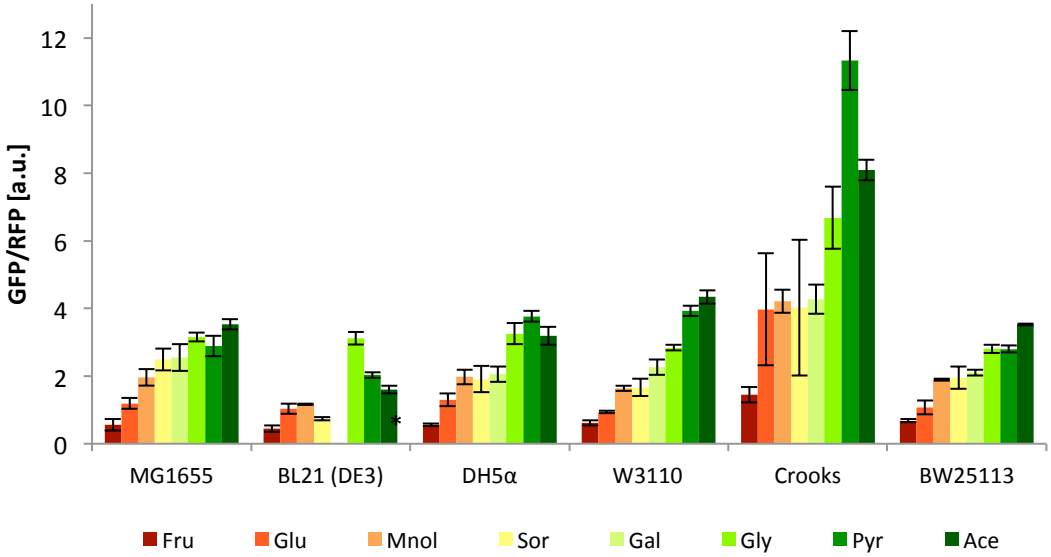


Figure 2.4) **GFP/RFP ratios of six commonly used *E. coli* strains.** The cells were grown in M9 minimal medium with different carbon sources. The fluorescence signals of GFP and RFP were measured by flow cytometry for 10,000 cells per run. The bars show the average of the mean GFP per RFP values of three independent experiments.
Abbreviations: Fru: Fructose, Glu: Glucose, Mnol: Mannitol; Sor: Sorbitol; Gal: Galactose; Gly: Glycerin; Pyr: Sodiumpyruvate; Ace: Sodiumacetate.
**) BL21 (DE3) is unable to grow on galactose.*

2.4.4 Applying pFlux in a mevalonate production strain

After testing the sensitivity of the Cra biosensor to different fluxes and its function in a variety of *E. coli* backgrounds, its applicability in a production strain was assessed. One of the most important possible applications for the glycolytic flux biosensor is in the development and optimization of production strains. The example that was chosen for the evaluation is the mevalonate production via the improved mevalonate pathway from Martin *et al*, 2003. The mevalonate pathway is a very suitable example, as an increase in mevalonate production causes a drain of acetyl-CoA, resulting in an increase of the glycolytic flux. The pMavT plasmid (Martin *et al*, 2003) was transformed to the *E. coli* BW25113 wild type and Δ*cra* strain together with pFlux. As a control, we used the empty plasmid pZA1. The GFP per RFP ratio dropped in wild type cells grown in glucose expressing the genes of the mevalonate pathway to 70% indicating a higher flux through glycolysis, while the GFP per RFP ratio did not change in the Δ*cra* mutant (Figure 2.5). This result demonstrates that pFlux can be applied for glycolytic flux measurements in production strains, where the glycolytic flux exceeds the normally observed fluxes.

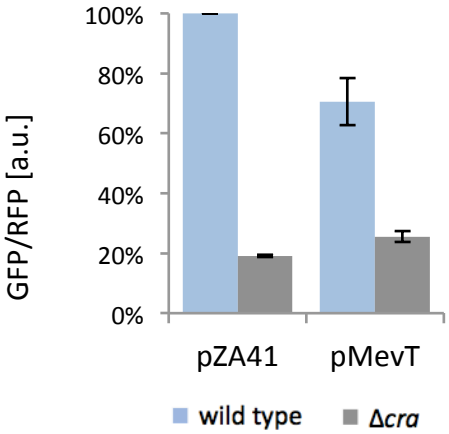


Figure 2.5) **The GFP per RFP ratios obtained by flow cytometry in absence (pZA41) and presence of the mevalonate pathway (pMevT).** The cells were grown in M9 medium containing 5 g/L glucose. The wild type (gray) and Δ*cra* strains (blue) were compared (n=3).

The integration of novel or enhanced biotechnologically relevant pathways often causes a change in the metabolic flux as for this example of mevalonate production. However, lower glycolytic flux rates can also be found in production strains. For instance during lysine production the carbon flux directed through the pentose phosphate pathway leading to a reduced glycolytic flux (Kiefer *et al*, 2004).

This characteristic of production altering glycolytic flux enables a broad range of possible applications for this glycolytic flux sensor, as the monitoring of flux changes might indicate higher production, in case no product sensor is yet available, or it could otherwise be integrated in a synthetic pathway control mechanism (Xu *et al*, 2014).

2.5 Summary

By changing the promoter region compared to the initial study of Kochanowski et al. and combining the Cra-controlled and constitutive expression of a reporter on one plasmid via two different fluorescent proteins, it was possible create a glycolytic flux sensor that can be used to compare the metabolic flux of different, individual cells via flow cytometry. Growing *E. coli* in presence of different carbon sources and measuring the GFP/RFP signal ratio demonstrated the dynamic range of the biosensor over the physiologically relevant range. By introducing the pMevT plasmid, it could furthermore be shown, that pFlux can also be used to measure glycolytic fluxes above the flux occurring during the growth on glucose.

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Appendix – Chapter 2

Table SII) **DNA oligo sequences for cloning.**
Abbreviations: -p: promoter region; BSsc: scrambled Cra-binding sit

Promoter regions		
PC028	AACCATUAATACCGCCATTTGG	*pfkA*-p forw
PC029	AGTTCTTCTCCUTTGCTCATGACTACCTCTGAACTTTG	*pfkA*-p rev
PC033	AACCATUCAACGCTGGGATC	*ppsA*-p forw
PC048	AGTTCTTCTCCUTTGCTCATCGAACAATCCTTTTGTG	*ppsA*-p rev
PC035	AACCATUGACGTAAATTCCTG	*ppc*-p forw
PC049	AGTTCTTCTCCUTTGCTCATATTACCCCAGACACC	*ppc*-p rev
PC063	ATTACTGGGUCGACGTTTTTTTCATCCGGT	*ppsA*-p BSsc forw
PC064	ACCCAGTAAUAGTCATATATTTTTTTACTTTTTAAGAC	*ppsA*-p BSsc rev
Genes		
PC019	AGGAGAAGAACUTTTCACTGGAG	*gfp* forw
PC021	ATTTGUAGAGCTCATCCATGCC	*gfp* rev
PC069	ACGACCUGCAGGGAGCAG	*mCherry* w term rev
PC070	ATGGTTUCCAAGGGCGAG	*mCherry* forw
Backbone assembly		
PC004	AATGGTUTCTTAGACGTC	p15A rev 1
PC031	ACAAAUGATAGAGGCATCA	p15A forw 1
PC055	ACTCTUCCTTTTTCAATATT	p15A forw 2
PC073	AACTGUACAACTTATATCGTATGGG	p15A forw 2
PC057	AAGAGUATGCCTCGGGCA	Sm/Sp resist. forw
PC060	ACAGTUATTTGCCGACTAC	Sm/Sp resist. rev
PC065	AAACCAUCGAACAATCCTTTTGTG	p15A forw 3
PC071	AGGTCGUGTCCTACTCAGGAG	p15A rev 3
PC072	AATGGTUGCGCTAGCGGAG	p15A forw 4

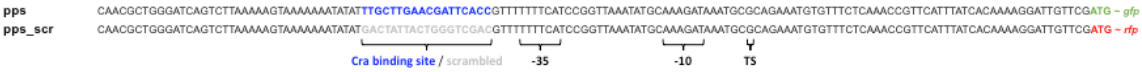
Table SIII) **Plasmid list.**
Abbreviations: *-p*: promoter region; BSsc: scrambled Cra-binding site

Name	**Description**	**Source**
pZA11MCS	p15A ori; ampicillin res. (amp), $P_{LtetO-1}$ promoter, MCS	(Lutz, 1997)
pZA14MCS	p15A ori; spectinomycin res. (spec), $P_{LtetO-1}$ promoter, MCS	this study
pGFPppsA	pZA4 + *ppsA-p_*gfp	this study
pGFPppc	pZA4 + *ppc-p_gfp*	this study
pGFPpykF	pZA4 + *pykF-p_gfp*	this study
pFlux	pZA4 + *ppsA-p_gfp* + *ppsA*-BSsc*_mCherry*	this study
pMevT	Mevalonate production pathway	(Martin *et al*, 2003)

Table SIV) **DNA sequences of the promoter regions.** The Cra binding site is highlighted in green, the -35 region in dark gray, the -10 region in light gray and the transcription start site in blue.

ppsA promoter region
CAACGCTGGGATCAGTCTTAAAAAGTAAAAAAATATATTTGCTTGAACGATTCACCGTTTTTTTCATCCGGTTAAATATGCAAAGATAAATGC
GCAGAAATGTGTTTCTCAAACCGTTCATTTATCACAAAAGGATTGTTCG
ppc promoter region
GACGTAAATTCCTGCTATTTATTCGTTTGCTGAAGCGATTTCGCAGCATTTGACGTCACCGCTTTTACGTGGCTTTATAAAAGACGACGAAAA
GCAAAGCCCGAGCATATTCGCGCCAATGCGACGTGAAGGATACAGGGCTATCAAACGATAAGATGGGGTGTCTGGGGTAAT
pykF promoter region
CGTAACCTTTTCCCTGGAACGTTAAATCTTTGATAACAATTTATTGTCTAACAAGTTGTATATTTTTTGAAACGCTGTTTTTGTTTTCCTTTTGGA
TTAATTTCAGCGTATAATGCGCGCCAATTGACTCTTGAATGGTTTCAGCACTTTGGACTGTAGAACTCAACGACTCAAAAACAGGCACTCACG
TTGGGCTGAGACACAAGCACACATTCCTCTGCACGCTTTTTCGATGTCACCTATCCTTAGAGCGAGGCACCACCACTTTCGTAATACCGGATTC
GCTTTCCGGCAGTGCGCCCAGAAAGCAAGTTTCTCCCATCCTTCTCAACTTAAAGACTAAGACTGTC

Table SV) DNA sequence of the pps and pps_scr promoter. TS= transcription start site



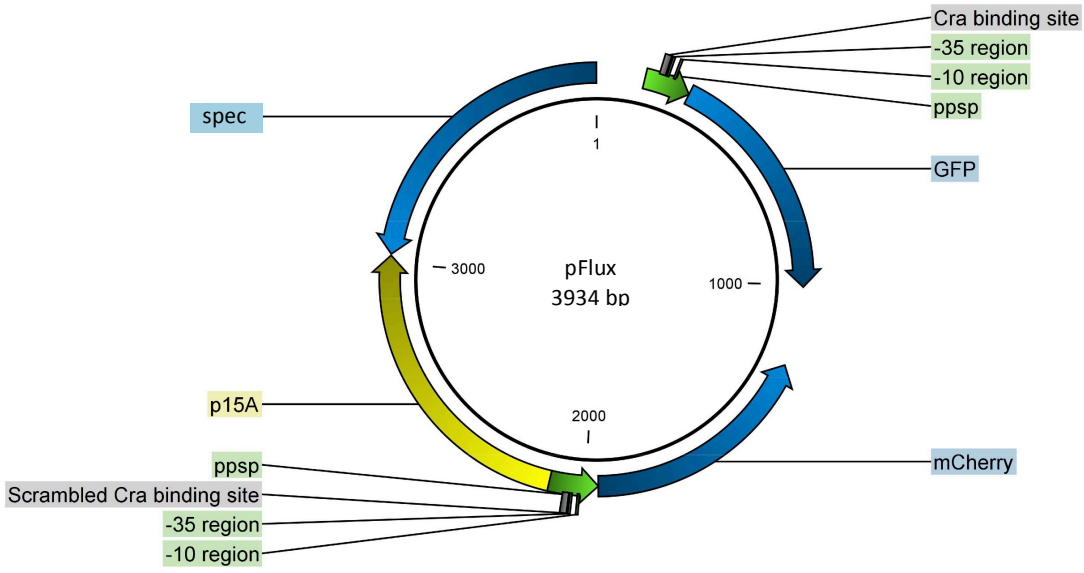


Figure SI) Plasmid map of pFlux.

CHAPTER 3 APPLICATION OF THE CRA BIOSENSOR

3.1 Abstract

Understanding the genetic factors that influence the metabolic flux of bacterial cells is of fundamental importance to bacterial physiology as well as *in silico* modelling and metabolic engineering. In this chapter, the novel genetically encoded glycolytic flux biosensor based on the *ppsA* promoter of *E. coli* is applied to screen for metabolic flux changes in a fast and parallelizable way. FlowSeq, the combination of fluorescence activated cell sorting (FACS) and deep sequencing, enabled the analysis of a library of 2,126 gene knockouts in *E. coli.* On a single cell level their individual effects on the glycolytic flux during the growth on galactose were studied. FACS was used to select for sub-populations of the full mutant library with a particular high or low flux phenotype, respectively. The subsequent deep sequencing gave detailed information of the distributions of individual deletion mutants in the subpopulations, linking the glycolytic flux phenotype to the genotype. After filtering and analysing the obtained data, one mutant (Δ*rpiA*) with a high-flux phenotype and 158 mutants with a low-flux phenotype were identified. The genes with the low flux phenotypes comprised genes of the glyoxylate cycle, galactitol metabolism and flagella synthesis. The glycolytic flux biosensor enhances our understanding of microbial physiology and can be applied to improve microbial cell factories.

3.2 Introduction

Biosensors have been applied in several high-throughput screens proving their relevance to enzyme engineering and cell factory optimization (Binder *et al*, 2013; Mustafi *et al*, 2012; Michener *et al*, 2012; Schendzielorz *et al*, 2014; Siedler *et al*, 2014b; Raman *et al*, 2014; Taylor *et al*, 2015). However, biosensors have not yet been used to study cellular states such as the glycolytic flux state of the cell. In the previous chapter, it could be shown that the novel pFlux Cra biosensor is capable of sensing the glycolytic flux over a physiologically relevant range and even above, as was shown in the example of the artificial mevalonate pathway.

In this chapter, the Cra biosensor is applied in a large-scale screen for single gene knockouts, which have a glycolytic flux altering effect in *E. coli*. With established techniques, like ^{13}C measurements the obtained fluxes are very precise and give a detailed, global picture of the metabolism, but it is not scalable to a whole genome screen (Zamboni *et al*, 2009; Fischer *et al*, 2004; Haverkorn van Rijsewijk *et al*, 2011). The aim of this genome-wide screen is to identify genes with flux-regulating properties that have not been identified yet.

3.3 Materials and methods

3.3.1 Strains, plasmids and DNA-oligos

Strains, plasmids and primers are listed in Table SII and

Table SIII. In the experimental setup, the used *E. coli* wild type and knockout strains derive from the KEIO collection (Baba *et al*, 2006). Primers and other oligos were ordered from IDT and were optimized for USER cloning. The adapters and primers for the Illumina sequencing were additionally HPLC purified and contained in the case of the UAD_tail a 3'-phosphorothioate bond and a 5'-phosphate for the barcoded sequencing adapters.

3.3.2 Cultivation and growth media

The growth media used in this study are Luria-Bertani (LB) complex medium, Super Optimal broth with Catabolite repression (SOC) medium and M9 minimal medium (Kochanowski *et al*, 2013) supplemented with filter-sterile trace element solution, resulting in a final concentration of 6.3 µM $ZnSO_4$, 7.0 µM $CuCl_2$, 7.1 µM $MnSo_4$, 7.6 µM $CaCl^2$ and 60 µM $FeCl_3$). The M9 medium contained 5 g/L of galactose. The LB and M9 media were supplemented with either 50 µg/mL kanamycin for the library preparation or 25 µg/mL spectomycin after transformation with pFlux.

If not stated otherwise, the cells were cultured in 5 mL medium in 15 mL cultivation tubes or 2 mL in 24-deep well plates. The cultivation tubes were incubated at 37°C, 190 rpm in a common shaking incubator and the 24-deep well plates at 37°C and 900 rpm on a tabletop plate shaker (Titramax 1000 incubator, Heidolph Instruments GmbH, Germany).

Strains were stored in 15 % v/v glycerol at -80°C.

3.3.3 KEIO library generation and plasmid transformation

For the library screen, the KEIO collection was pooled. In order to obtain the best possible coverage, the individual strains were plated from the glycerol stock on LB agar plates with 50 µg/mL kanamycin. The plates were incubated over night at 37°C and the colonies washed off with 1 ml liquid LB medium without antibiotics. 100 µl of each cell suspension was used to inoculate 150 mL LB medium containing 50 µg/mL kanamycin in one 500 mL shaking flask. The resulting inoculation volume for the 150 mL medium was in 5.6 ml in total. The flasks were incubated at 37° C, 190 rpm until OD 0.5 was reached. In order to prepare the cells for electroporation and to remove all traces of salts, the cells were pre-chilled on ice for 10

minutes and afterwards washed three times with ice-cold 10 % (v/v) glycerol. Between each washing step, the cells were pelleted for 5 minutes at 4000 rpm and 0°C and the supernatant discarded. The first two washing step were carried out in a 50 ml Falcon tube with 50 ml and 25 ml cell suspension, respectively. The last washing step was carried out in a 2 ml reaction tube and pelleted in a pre-chilled tabletop centrifuge. The pellet was resuspended in 200 µl of the ice-cold 10 % (v/v) glycerol. 50 µl of this cell suspensions were transferred to a pre-chilled 1 mm electroporation cuvette and 100 ng of pFlux added. The cells were electroporated at 1.8 kV and resuspended in 950 µL pre-warmed SOC medium. After transferring them to a 1.5 ml reaction tube, they recovered for 1 h at 37°C and 500 rpm. The cell suspension was used to inoculate 50 ml LB medium (additionally added 0.5 mM MgSO4 and 25 µg/ml kanamycin) in a 250 ml shaking flasks. The cultures grew over night at 37°C and 190 rpm. 700 µl of the overnight culture were diluted to 15 % glycerol stocks and stored at -80°C until use.

3.3.4 Fluorescence Activated Cell Sorting (FACS)

For the individual cell sorting rounds, 50 ml LB medium containing 25 ng/mL spectomycin were inoculated with 1 ml of the KEIO cryo stocks in order to maintain diversity. The cultures were incubated at 37°C, 190 rpm over night. 50 ml M9 medium containing 5 g/L glucose or galactose and the respective antibiotics were inoculated from the LB precultures to an OD_{600} of 0.01. The cultures were incubated at 37°C, 190 rpm over night. Fresh M9 medium was inoculated to an OD_{600} of 0.05. After 4 hours of shaking incubation, samples were taken and diluted in FACSFlow (BD) in order to prepare them for sorting FACSAria (BD) with a 488 nm and 561 nm LASER. To collect the GFP and RFP signals, the FITC (530/30) and PE-Texas Red (610/20) filter were used. The individual cells were sorted according to their signal in the FITC (GFP fluorescence) and PE-Texas Red (RFP fluorescence) channels. One percent of the outliers towards a higher, respectively lower FITC per PE-Texas Red signal ratio were collected (Figure 3.1). The cells were sorted into 12 cm FACS tubes with 1 ml LB medium (25 ng/ml spectomycin) and grown at 37°C, 190rpm over night. The cells were pelleted at 4,500 rpm and stored at -20°C.

3.3.5 Genome purification, amplification and sequencing

Library preparation and validation closely followed the TnSeq protocol of Lennen *et al.* (Lennen & Herrgård, 2014). To adjust the protocol to the KEIO strains, the biotinolated PCR primer was designed to match the 19 base pair long FTR scar (GAAGCAGCTCCAGCCTACA) that

was left from the deletion process to generate the knockout library (Baba *et al*, 2006). In order to amplify the respective knockout regions a biotinolated primer (/5BiotinTEG/AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGCAGCTCCAGCCTACA) and a standardized UAD-tail primer (GATCTACACTCTTTCCCTACACGACG) was used. The barcoded adapter matched the Illumina NexTera platform. The sequencing was performed on an Illumina miSeq, 150 bp, running 1 pM of DNA per sample.

3.3.6 Data analysis

In order to analyze the obtained sequence reads from the miSeq, we run a customized script, consisting of data preparation, quality check, creating database of quality reads, searching for *E. coli* genes using their bar codes and summarize the results in a table.

The PCR amplification of the region following the FRT scar allows evaluating the occurrence of a deletion mutant in the different pools without mapping it to the entire genome. Instead, it can be simply matched to the list of primers Baba et al. (Baba *et al*, 2006) used to generate the knockout strains. The circumventing of the mapping results in less bias and a clear output list of each gene and the number of annotated reads.

In the data preparation process, the short-read .fastq files were converted into .fasta files and a blast database build for each experiment as well as a tabular file with reads indexed by their read identification number. The quality check assured that only reads that began with the FRT specific DNA sequence GAAGCAGCTCCAGCCTACA were taken into consideration and blasted (blastn) considering the parameters given in Supplementary Table SII to search for small sequences. We then extract the reads that have at least 80% coverage of the primers with a maximum of two mismatches and discard those that do not meet these criteria. The extracted reads form the blast database that is used to search for the barcodes. The barcode list is based on the reverse primers that were used by Baba *et al*. (Baba *et al*, 2006) to delete the respective genes. For each barcode, corresponding to one gene deletion, the number of reads in the blast database is counted they match, allowing a maximum of three mismatches.

In order to reduce possible bias in the analysis, we normalized the reads in two steps. In the first step, the numbers of reads for every gene in each sequencing run was normalized on the over-all number of reads of the run to make the results of the different sequencing runs comparable. In the second step, the number of reads of the sorted population was divided by the number of reads of the unsorted population, which was grown under the same conditions.

The second normalization step was performed to reduce the bias of unlike growth rates of different mutants and to obtain the fold enrichment of the different pools compared to the not enriched population. The threshold for consideration of a gene was set to a minimum of 10 annotated reads in the library.

3.3.7 Clustering and GO analysis

The enrichment and depletion of the individual genes in the different pools were clustered by their likeliness, generating ten clusters (Figure SIII; Table SVII; Table SVIII; Table SIX).The ten clusters were subsequently grouped into three groups. Clusters with genes enriched in the low flux pools and depleted in high flux were grouped, as well as all clusters with no apparent enrichment profiles. In case of a high flux phenotype, there was only one cluster where genes would be enriched in the high flux pools and depleted in the low flux pools. A GO analysis based on their biological function was performed with the genes of the group with a low flux phenotype (Ashburner *et al*, 2000; The Gene Ontology Consortium, 2014).

3.3.8 Growth rate characterization

The gene deletions that showed an interesting phenotype in the flux data analysis were tested individually on their growth rate.2 ml of LB medium (25 nm/ml Kanamycin) in a 24-deep well plate was inoculated with strains from a cryo stock and grown at 37°C and 1000 rpm in a tabletop plate shaker (Titramax 1000 incubator, Heidolph Instruments GmbH, Germany) until exponential or stationary phase. The cells were subsequently diluted 1:50 in M9 medium containing either 5 g/L glucose or 5 g/L galactose and grown at the same conditions over night. The cells were diluted 1:200 in fresh M9 media containing the respective carbon source. 200 µl of the fresh culture were transferred to a micro titer plate. The plate was sealed with Breathe-Easy sealing membrane (Sigma-Aldrich). The OD_{630} was measured in a plate reader over a period of 16 h and the growth rate determined.

3.4 Results and discussion

The glycolytic flux biosensor plasmid pFlux was transformed into a library of the KEIO collection, a collection of non-lethal single gene deletions in *E. coli* (Baba *et al*, 2006). The library of knockout strains was grown in M9 minimal media containing 5 g/L galactose. Galactose was chosen as carbon source, as it takes the same glycolytic route as glucose but with a lower flux rate (Haverkorn van Rijsewijk *et al*, 2011). As it was shown in the previous chapter, galactose as a carbon source enables to show as well an increase in signal as well as a decrease, respectively indicate a higher and lower glycolytic flux (Figure 2.3). During growth on glucose, Cra is furthermore mostly inactive, thus we assume that choosing galactose as carbon source also offers the possibility to give a better sensitivity for changes towards higher flux.

Single cells were sorted according to their flux phenotype by fluorescence activated cell sorting (FACS). After recovery in LB medium, the gene regions downstream of the flippase recognition target (FTR) sequence was amplified and sequenced (Figure 3.1A,B). Each gene knockout of the KEIO collection still harbors the last 21 bases of the deleted gene, enabling an easy and rather straightforward possibility of matching the amplified gene regions to the respective gene deletions. The biological replicates showed a good agreement (R^2 0.87 – 0.99) (Figure SII) and the 1% and 5% pools on each side of the dot plot have a huge overlap (Figure 3.2). A total of 2,126 individual deletion mutants were found in the library after growth in minimal medium with galactose, which resembles more than 56 % of the whole KEIO collection. We assume that those knockout mutants that do not appear in the library had either not been transformed with pFlux or had too high fitness defect and were outcompeted by the other strains during the initial growth of the cell library (Cao et al, 2014). After coverage and quality filtering, which is explained in chapter 3.3.6, 504 genes remained. The 504 genes were analyzed according to the similarity of their enrichment and depletion patterns in the different pools compared to the total, unsorted library (Figure 3.2). 3 gene deletions (Δ*ompC*, Δ*rpiA* and Δ*ynfH*) were at least two times enriched in the high flux pools, while also depleted in the low flux pool (Table SIX). OmpC is a porin in the outer membrane and forms non-specific pores, which allow the diffusion of small hydrophilic molecules across the outer membrane (Heller & Wilson, 1981) whereas YnfH is considered a subunit of a putative selenite reductase (Guymer *et al*, 2009). The deletions of *ompC* and *ynfH* have been shown to give *E. coli* a growth benefit compared to the wild type in presence of antibiotics. In the case of OmpC, it was tested with antibiotics of the ß-lactam family (Liu *et al*, 2012) and in the case of ΔynfH

spectomycin directly (Vlasblom *et al*, 2015). Hence, the higher flux phenotype of Δ*ompC* and Δ*ynfH* mutants in the presence of antibiotics compared to the overall knockout library and the wild type strain can be explained by their advantages for growth in presence of antibiotics (Table SX).

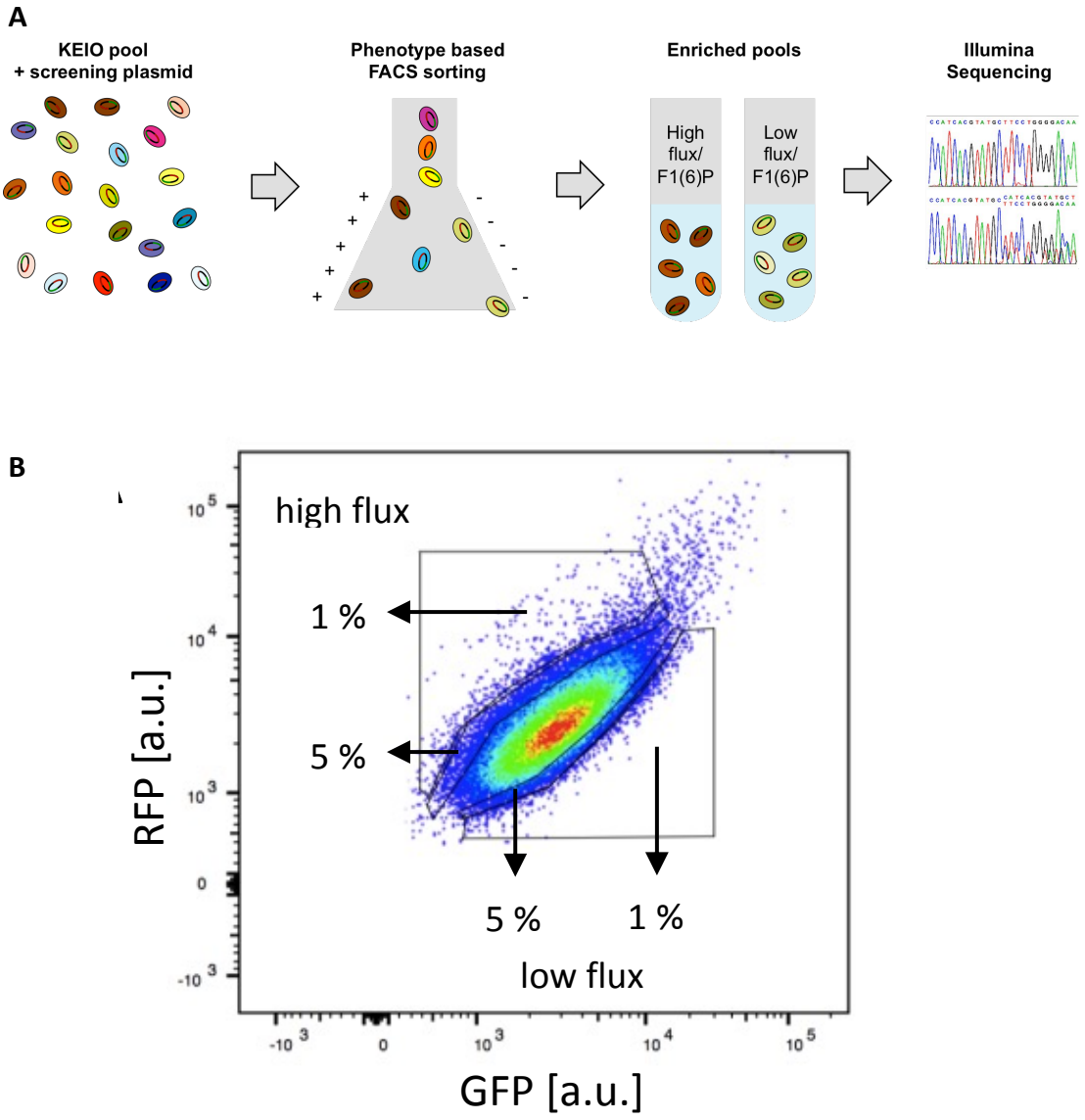


Figure 3.1) **FlowSeq workflow**. **(A)** Overview of the FACSSeq procedure. In the first step, the deletion library is transformed with pFlux, followed by FACS and recovery in LB medium. In the last step, the purified and enriched gene knockout regions are sequenced. **(B)** Dot plot of the RFP and GFP signals of the KEIO library grown in 5 g/L galactose. The four shown gates were used to sort the cells for their flux phenotype, sorting 100,000 cells into the 1% and 5% gates. Additionally, a sample of 1,000,000 cells was collected, to determine the genetic composition of the total population at the point of sorting.

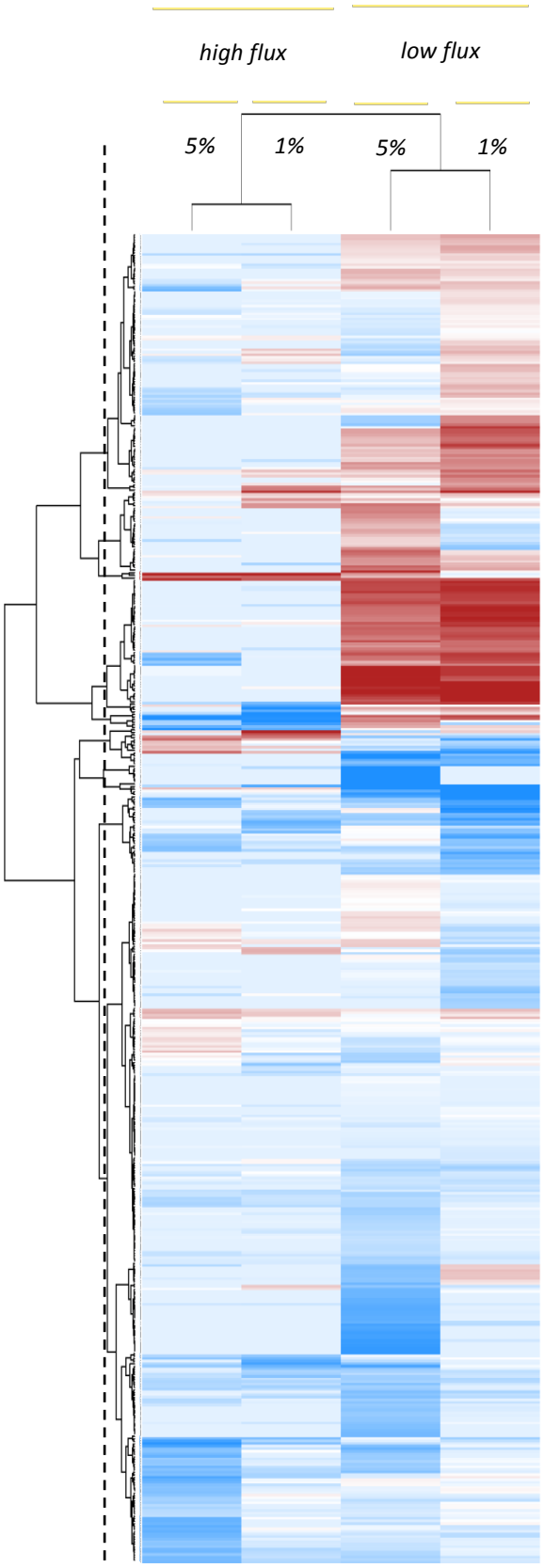


Figure 3.2) **Clustered heatmap of the enriched and deprived genes of the different glycolytic flux phenotype pools, compared to the total knockout library.** Enrichment is shown in red and depletion in blue. The genes are sorted according to their similarity in enrichment and depletion patterns, resulting in the cluster tree shown on the left. The dotted line indicates the complexity level that was used in the further analysis as the definition point for the different clusters.

The remaining gene knockout strain with a significant high flux phenotype is Δ*rpiA*, which encodes for the ribose-5-phosphate isomerase A. RpiA catalyzes the first step of the non-oxidative phase of the pentose phosphate pathway (PPP) and hence the step towards the biosynthesis of nucleotides and aromatic amino acids (Skinner & Cooper, 1971). We compared the growth rates in a plate reader of Δ*rpiA* and the wild type strain on minimal medium with glucose or galactose supplemented and could detect a significant growth difference between Δ*rpiA* and the wild type on galactose (Δ*rpiA* 0.308 ± 0.005 h^{-1} and wt 0.223 ± 0.006 h^{-1}), and a still observable advantage on glucose ((Δ*rpiA* 0.416 ± 0.052 h^{-1} and wt 0.362 ± 0.007 h^{-1}). These findings are surprising as a Δ*rpiA* mutant should not be able to grow on glucose (Sørensen & Hove-Jensen, 1996) and possibly not on galactose. It is known that the isoenzyme RpiB is capable of supplementing RpiA in a deletion strain, but the expression needs to be induced by e.g. ribose and had been shown to be not active on glucose (Sørensen & Hove-Jensen, 1996). It was generally considered a substituting enzyme of minor function, but recent studies of Δ*rpiB* deletion strains have found surprisingly strong effects on biomass production. Kim and Reed found that the Δ*rpiB* mutant had a 30 % decrease in biomass yield compared to the parental strain (Kim & Reed, 2012). In regard to our findings, this could mean that the Δ*rpiA* mutant has an up-regulation of RpiB expression, resulting in thus far uncharacterized positive effects on the glycolytic flux. This hypothesis will need experimental evidence, but it makes the isoenzymes RpiA and RpiB very interesting targets for further research on glycolytic flux control and optimization.

To conclude, only one of the three most prominent knockout mutants with a high flux phenotype could actually be an interesting candidate for further flux studies. The other two candidates are having higher resistance against spectinomycin and hence a growth advantage compared to the other strains. In total 158 genes were found to be connected to a low flux phenotype, significantly depleted in the high GFP/RFP fraction and enriched in the low GFP/RFP fraction (Figure 3.2, Table SVIII) We could identify the deletion mutants of the transcriptional regulators GalR and GalS in this fraction as positive controls. GalR and GalS take part in the regulation of operons involved in transport and catabolism of D-galactose in presence of high galactose concentrations and under glucose limitation (Semsey *et al*, 2007; Weickert & Adhya, 1992a). It is expected that the deletion of these genes leads to a drastic reduction of the glycolytic flux when grown on galactose and therefore can be seen as a validation of the applicability of the glycolytic flux biosensor in this experimental setup.

Additionally, a GO analysis of the 158 genes with the low flux phenotype was performed. A GO analysis is helpful to give a global picture on which groups of genes with biologically related

functions are significantly enriched or depleted in a dataset, compared to the statistical expectation. In detail, the corresponding data file for this GO analysis contains all genes of the *E. coli* K12 genome, grouped based on their biological function. An initial step uses the observed gene coverage to calculate, how many genes are expected per functional group, if the distribution was entirely random. In a second step, these expected numbers are compared with the actual detected numbers of each group and the fold enrichment, compared to expected count, and the statistical relevance is calculated. (Ashburner *et al*, 2000; The Gene Ontology Consortium, 2014)

The GO analysis revealed that genes related to flagella machinery (7 genes out of 26) are enriched more than five-fold compared to what is expected ($\rho = 7.77x10^{-06}$) in the low-flux phenotype clusters. Flagella are mainly responsible for chemotaxis and hence enable the individual cells to move in direction of higher carbon source concentration (Eisenbach, 2007). Even though chemotaxis is necessary for the cells to obtain nutrients, this ability is not as relevant in the continuous mixing conditions of this experiment. As flagella are a very expensive machinery and compete with transporters for the proton gradient over the cell membrane, it was shown previously that its expression is down regulated in glucose-limited fed-batch cultivation (Lemuth *et al*, 2008). Hence, deleting parts of the chemotaxis machinery reduces the metabolic burden that is put upon the cell assumingly lowering the glucose drain and hence the glycolytic flux (Martínez-García *et al*, 2014).

Another metabolic function significantly enriched was the galactitol metabolic pathway (4 genes out of 7 in the *E. coli* genome, $\rho = 4.63x10^{-05}$). Gene deletions include *gatA* and *gatC*, subunits of the galactitol/sorbitol PTS permease. Either, the transporter is also accepting galactose to some extend and increasing the intracellular galactose concentration and subsequently a deletion reduces the uptake and hence the flux, or galactitol, an alcohol of galactose, is potentially present as a byproduct in the galactose solution taken up by the cells and resulting in a higher overall flux.

The third enriched GO-pathway was the glyoxylate pathway, including all necessary genes (Δ*aceA*, Δ*aceB* and Δ*aceK*) in the low flux phenotype. *AceA* and *aceB*, coding for isocitrate lyase and malate synthase, respectively, are the two enzymes needed for a functional glyoxylate pathway. AceK controls the branch point between the TCA cycle and the glyoxylate cycle, by phosphorylation of the isocitrate dehydrogenase (ICD) and thereby modulation of the ICD activity (LaPorte & Koshland, 1982; Cortay *et al*, 1988). A deletion of *aceK* results in constant activation of the ICD and reduced glyoxylate pathway activity. As it was shown in previous ^{13}C metabolic flux analysis (Haverkorn van Rijsewijk *et al*, 2011), the glyoxylate shunt is having a

much stronger flux when *E. coli* is grown on galactose compared to glucose. The genes involved in this process lead to an overall reduction of glycolytic flux when those cells are grown on galactose.

It would be expected to find a large number of gene deletions related to the central carbon metabolism, since many of these have a naturally huge impact on the glycolytic flux. However, gene deletions in the central carbon metabolism are often lethal (e.g. *eno*, *pgk*, *gapA*) and therefore not present in the KEIO collection (Baba *et al*, 2006). Moreover, even if the deletion is not directly lethal, it still results in a strong growth defect. Thus, those knockout strains do not appear in the library (e.g. *pgi*, *pfkA*, *tpiA*), resulting in the decreased library size and the low number of genes related to central metabolism appearing in the data sets.

Interestingly, many of the 158 gene deletion mutants in this low flux phenotype cluster are either of unknown and putative functions or phage and prophage related. It is a very remarkable finding that the deletion of phage and prophage genes results in an actual decrease in glycolytic flux. It was previously shown that prophage genes are not always only a silent cargo in the bacterial genome but can be highly expressed and change their expression patterns in different conditions, especially in pathogens (Smoot *et al*, 2001). This might be related to the fact that prophage genes have been identified to give their host certain advantages, even in the case of the laboratory strain *E. coli* K-12 (Barondess & Beckwith, 1990). The expression of the prophage gene *bor* significantly increases the survival of *E. coli* K-12 on animal serum. Thus, it might very well be that these proteins give *E. coli* advantages that are not yet described but would be very interesting to study further. The appearance of the large number of genes with unknown and putative function is also interesting, as it highlights again that there is still a large, so far uncharacterized, regulatory network in place. This observation matches the recent findings of Hutchison et al. (2016). They could show that 473 genes are required for a viable, synthetic minimal genome. Of those 473 genes, 149 genes are of unknown function. Even though the exact function of these 158 gene knockouts is still unknown, this list provides an interesting starting point for further, more targeted studies.

In regard to biotechnological applications, the identified gene knockouts with a low flux phenotype are very interesting. These deletion strains showed a lower flux phenotype, but were not outcompeted by growth rate. Therefore, these mutants could provide an additional flux span that can be redirected towards the production process.

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Appendix – Chapter 3

Table SVI) blastn parameters considered in the analysis of the FlowSeq data

Parameter	Value	Comment
word_size	4	To match small sequences
evalue	1000	To match small sequences
num_threads	28	To fully utilize the machine
max_target_seqs	Exp_read_size	Number of reads in Experiment: to get as much as possible of the reads

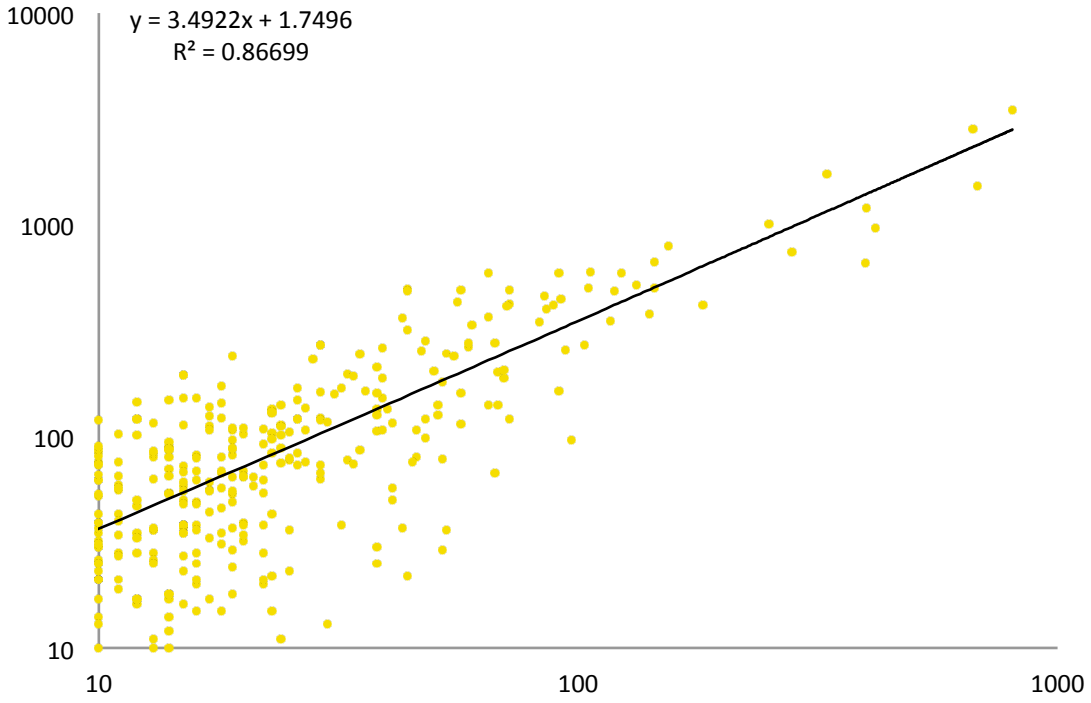

Figure SII) Log scale dot plot of the individual gene coverage for the duplicates of the 1 % low flux pool. Each dot represents one gene. The plot shows the raw numbers before normalization. A linear regression line is presented with a R^2 of 0.86699.

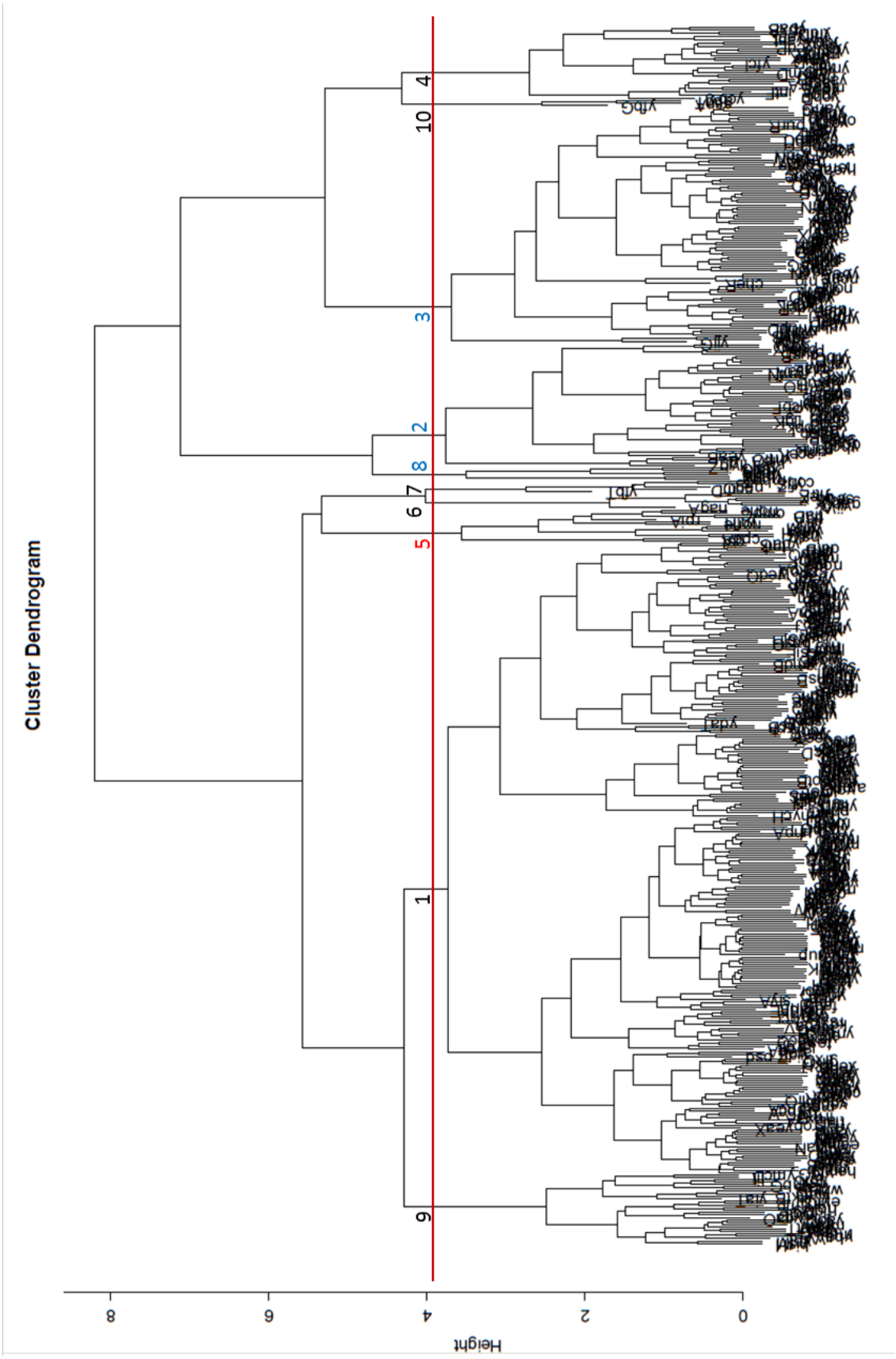

Figure SIII) Dendrogram of the different clusters. A threshold was set to identify 10 different sub clusters (1-10). Clusters representing a high flux phenotype are shown in red (5), and clusters including deletion mutants with a low flux phenotype are shown in blue (2, 3 and 8).

Table SVII) Cluster of unchanged genes, ordered by gene name. Clusters derived from dendrogram in Figure SIII.

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
Cluster 1						
adhE	b1241	Enzyme; Energy metabolism, carbon: Fermentation	0.42	-0.75	-0.66	0.44
aer	P50466	Regulator; Degradation of small molecules: Carbon compounds	0.18	-0.96	-0.23	-0.96
alsC	P32720	Putative transport; Not classified	0	-1.95	0	0
ampD	P13016	Regulator; Not classified	0	-1.65	0	0
ansB	P00805	Enzyme; Degradation of small molecules: Amino acids	0.17	-1.18	-0.31	-0.1
araE	P0AE24	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	-0.57	-0.16	1.27	0.86
aroG	P0AB91	Enzyme; Amino acid biosynthesis: Phenylalanine	0	-0.14	0.03	0.92
arsB	P0AB93	Transport; Drug/analog sensitivity	0.42	-0.23	-0.01	-0.55
arsC	P0AB96	Enzyme; Drug/analog sensitivity	-0.06	-0.91	-0.55	-0.78
artQ	P0AE34	Transport; Transport of small molecules: Amino acids, amines	-0.51	0	0	0
asnA	P00963	Enzyme; Amino acid biosynthesis: Asparagine	0.02	-0.65	-0.47	-1.01
atpA	P0ABB0	Enzyme; ATP-proton motive force interconversion	-0.02	-0.64	0.06	0.01
bcr	P28246	Transport; Drug/analog sensitivity	0.34	0.31	0.37	0.89
bcsC	P37650	Putative enzyme; Not classified	0.03	0.46	0.24	-1.5
bcsG	P37659	Null	0.39	-0.16	-0.75	-1.26
bdm	P76127	Null	-0.05	-0.24	0	-1.31
betB	P17445	Enzyme; Osmotic adaptation	0.33	-0.12	0	-1.58
cadA	P0A9H3	Enzyme; Degradation of small molecules: Amino acids	0	-2.13	0	0
caiE	P39206	Putative enzyme; Central intermediary metabolism: Pool, multipurpose conversions	0	0.45	0	0
ccmC	P0ABM1	Transport; Protein, peptide secretion	-0.81	0	0	0
chiA	P13656	Putative enzyme; Not classified	-0.56	0.11	0	0.09
citA	P77510	Regulator; Degradation of small molecules: Carbon	1.32	0.28	0.44	1.25

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
citB	P0AEF4	Regulator; Degradation of small molecules: Carbon compounds	0	-0.97	0	-1.39
citC	P77390	Enzyme; Central intermediary metabolism: Pool, multipurpose conversions	-0.15	-0.44	-0.1	-0.38
clcA	P37019	Transport; Not classified	-0.05	0.06	0	0
clpS	P0A8Q6	Null	0	-1.42	0	0
cpsB	P24174	Enzyme; Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	-0.12	-0.43	0.18	0.61
creC	P08401	Enzyme; Global regulatory functions	0	-2.23	0	0
csiE	P54901	Null	0.14	0.43	-0.37	-1.25
dadA	P0A6J5	Enzyme; Degradation of small molecules: Amino acids	-0.31	-0.31	-0.1	0.16
dapB	P04036	Enzyme; Amino acid biosynthesis: Lysine	0.03	0.07	0	0
ddlB	P07862	Enzyme; Murein sacculus, peptidoglycan	0	-0.93	0	-1.59
dsbA	P0AEG4	Enzyme; Proteins - translation and modification	0	-0.45	-0.29	0
eamA	P31125	Null	-0.21	0.46	0	0
ecnA	P0ADB4	Null	0.05	0.39	0.09	0.41
elbA	P75987	Null	-0.2	0	0	0
etp	P0ACZ2	Enzyme; Not classified	0.27	-0.44	0.24	0.8
eutQ	P76555	Putative enzyme; Not classified	-0.23	-1.21	-0.45	-0.61
eutT	P65643	Null	-0.31	-0.78	-0.09	-0.07
fabF	P0AAI5	Enzyme; Fatty acid and phosphatidic acid biosynthesis	-0.31	0	0	0
fadB	P21177	Enzyme; Degradation of small molecules: Fatty acids	0.53	-0.7	0	0.32
fepE	P26266	Transport; Transport of small molecules: Cations	0	0.63	0	0
fhiA	Q47153	Null	0	0.09	0	0
fimG	P08190	Structural component; Surface structures	0	-1.77	0	0
fkpA	P45523	Enzyme; Proteins - translation and modification	-0.45	-0.05	-0.69	-2.02
fliD	P24216	Putative structure; Surface structures	-0.36	-0.53	-0.82	-0.45
fliG	P0ABZ1	Structural component; Surface structures	0.47	0.06	0	-1.21
fliH	P31068	Transport; Surface structures	0.07	-0.01	0.12	0.09

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
fliQ	P0AC07	Putative structure; Surface structures	-0.75	-0.05	0.39	-0.46
fliZ	P52627	Structural component; Unknown	-0.03	-0.83	-0.81	-0.61
frmB	P51025	Enzyme; Formaldehyde degradation	-0.34	0.76	0	0.54
frvR	P32152	Putative regulator; Not classified	0.09	-0.23	-0.05	0.05
galM	P0A9C3	Enzyme; Central intermediary metabolism: Pool, multipurpose conversions	-0.42	-0.32	-0.11	-0.22
glnG	P0AFB8	Regulator; Amino acid biosynthesis: Glutamine	-0.74	0.22	0	0
gltX	P04805	Enzyme; Aminoacyl tRNA synthetases, tRNA modification	0.04	-0.64	-0.54	-1.47
gpp	P25552	Enzyme; Global regulatory functions	-0.52	-0.34	-0.09	-1.5
gspH	P41443	Putative transport; Not classified	-0.29	-0.76	-0.35	-0.37
gspJ	P45761	Putative transport; Not classified	0.3	-0.34	0.3	-0.89
gyrB	P0AES6	Enzyme; DNA - replication, repair, restriction/modification	-0.22	-0.53	-0.3	-0.38
hcr	P75824	Putative enzyme; Not classified	0.22	-0.74	-0.08	-1.07
hdeD	P0AET5	Null	0.21	0.01	-0.66	0
hemN	P32131	Enzyme; Biosynthesis of cofactors, carriers: Heme, porphyrin	0.17	0.74	0	0
hyaB	P0ACD8	Enzyme; Energy metabolism, carbon: Aerobic respiration	-0.3	-0.51	0	0
hybE	P0AAN1	Phenotype; Energy metabolism, carbon: Anaerobic respiration	0	-0.86	0	0
hybF	P0A703	Regulator; Energy metabolism, carbon: Anaerobic respiration	0	-1.78	0	0
hycF	P16432	Putative enzyme; Energy metabolism, carbon: Fermentation	0	0.95	0	0
hycH	P0AEV7	Factor; Energy metabolism, carbon: Fermentation	1.02	-1.16	0	-0.63
kdsC	P0ABZ4	Null	0.08	0	-0.16	0.59
lgt	P60955	Enzyme; Macromolecule synthesis, modification: Phospholipids	0.89	0.51	1.05	1.4
livM	P22729	Transport; Transport of small molecules: Amino acids, amines	0.4	0	0.08	-1.26

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
lsrD	P0AFS1	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	1.13	-1.17	0	0
lysC	P08660	Enzyme; Amino acid biosynthesis: Lysine	-0.14	-0.73	-0.18	-0.16
mdtA	P76397	Putative membrane; Not classified	0.06	-0.51	0.34	-1.6
menD	P17109	Enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	-0.02	-0.43	0.08	0.02
metL	P00562	Enzyme; Amino acid biosynthesis: Methionine	-0.96	-0.08	0	-0.31
mgsA	P0A731	Enzyme; Central intermediary metabolism: Pool, multipurpose conversions	-0.2	-0.42	0	0
mngA	P54745	Enzyme; Proteins - translation and modification	0	0.22	0	0
molR	P33345	Null	0	-0.52	0	0
mrcA	P02918	Enzyme; Murein sacculus, peptidoglycan	-0.54	-0.13	-0.33	-1.46
mrp	P0AF08	Putative enzyme; Not classified	0.3	-0.94	-0.2	0.2
msrA	P0A744	Enzyme; Proteins - translation and modification	-0.13	-1.14	0	0
murD	P14900	Enzyme; Murein sacculus, peptidoglycan	-0.11	-1.29	-0.59	-1.39
napD	P0A9I5	Null	0	-2.19	0	0
nikC	P0AFA9	Transport; Transport of small molecules: Cations	-0.22	-0.8	-0.65	-1.44
nlpB	P0A903	Membrane; Macromolecule synthesis, modification: Lipoprotein	0.14	-0.2	-0.08	-0.5
nrdD	P28903	Enzyme; 2'-Deoxyribonucleotide metabolism	0	0.12	0	0
pepQ	P21165	Enzyme; Degradation of proteins, peptides, glyco	-0.05	-1.29	0.35	-1.17
pflD	P32674	Enzyme; Energy metabolism, carbon: Anaerobic respiration	-0.02	-0.62	-0.4	-0.73
pfs	P0AF12	Null	-0.26	-0.52	0.12	-0.4
phnA	P0AFJ1	Null	-0.4	0	0	0
phnG	P16685	Enzyme; Central intermediary metabolism: Phosphorus compounds	1.13	-1.09	-0.05	-0.16
phoA	P00634	Enzyme; Central intermediary metabolism: Phosphorus compounds	0.63	-1.51	0	0
phoH	P0A9K1	Regulator; Central intermediary metabolism: Phosphorus compounds	0.34	0.03	-0.2	0.08

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
phoU	P0A9K7	Regulator; Global regulatory functions	-0.06	-0.48	-1.24	-2.33
pncA	P21369	Null	-0.74	1	0.73	0.32
potB	P0AFK4	Transport; Transport of small molecules: Amino acids, amines	-0.21	-1.7	-0.31	-0.28
psd	P0A8K1	Enzyme; Macromolecule synthesis, modification: Phospholipids	-1	-0.59	1.3	0
purH	P15639	Enzyme; Purine ribonucleotide biosynthesis	0.43	0.03	-0.12	-1.15
pyrB	P0A786	Enzyme; Pyrimidine ribonucleotide biosynthesis	-0.2	0	0	0
paaC	P76079	Enzyme; Phenylacetic acid degradation	0.1	-0.4	-0.65	-1.42
paaF	P76082	Enzyme; Phenylacetic acid degradation	0.15	-0.08	0.15	-0.22
qor	P28304	Enzyme; Energy metabolism, carbon: Electron transport	0.12	-0.23	-0.4	-0.27
racR	P76062	Phage or Prophage Related	0.1	0.35	0	0
radA	P24554	Putative enzyme; Degradation of proteins, peptides, glycopeptides	-0.64	-0.8	0	0
rbbA	P37624	Putative transport; Not classified	-0.21	-0.55	0.34	-0.23
rbsC	P0AGI1	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	-0.17	-0.58	-0.32	-0.69
rcsF	P69411	Regulator; Surface polysaccharides & antigens	0	-1.08	0	0
rep	P09980	Enzyme; DNA - replication, repair, restriction/modification	0.43	-0.28	0	-0.75
rffG	P27830	Enzyme; Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	0	0.46	0.09	0
rne	P21513	Enzyme; Degradation of RNA	-0.01	-0.39	-0.17	-1.14
rob	P0ACI0	Factor; DNA - replication, repair, restriction/modification	-0.64	0.4	0	0.45
rpe	P0AG07	Enzyme; Central intermediary metabolism: Non-oxidative branch, pentose pathway	0.01	0.15	-0.39	0
rph	P0CG19	Enzyme; Degradation of RNA	0.02	-0.54	-1.76	0
rplW	P0ADZ0	Structural component; Ribosomal proteins - synthesis, modification	-0.6	-0.66	-0.21	-0.33
rpmF	P0A7N4	Structural component; Ribosomal proteins - synthesis,	0.31	-0.87	-0.43	-1.49

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
rseC	P46187	Regulator; Global regulatory functions	0.2	0	0	0
rsxC	P77611	Putative membrane; Not classified	0	0.07	0	0.55
sbcD	P0AG76	Enzyme; Degradation of DNA	0	0	0	-0.25
selD	P16456	Enzyme; Aminoacyl tRNA synthetases, tRNA modification	0.25	0.52	0	0
sgcB	P58035	Null	-1.08	0	0	0
sgcQ	P39364	Putative enzyme; Not classified	0	-0.47	0.36	1.16
sgrR	P33595	Transport; Not classified	0	-1.42	0	0
slyA	P0A8W2	Regulator; Adaptations, atypical conditions	0.59	-0.03	-1.08	0.38
soxS	P0A9E2	Regulator; Global regulatory functions	0	0.54	0	0
speB	P60651	Enzyme; Central intermediary metabolism: Polyamine biosynthesis	0	-1	-1.14	0
tam	P76145	Enzyme; Detoxification	1	-1.27	0	0
tdcD	P11868	Putative enzyme; Not classified	-0.25	-2.17	0	0
tehB	P25397	Enzyme; Drug/analog sensitivity	-0.5	0.17	0	0
tesA	P0ADA1	Enzyme; Fatty acid and phosphatidic acid biosynthesis	1.19	0.73	0.89	1.27
tesB	P0AGG2	Enzyme; Fatty acid and phosphatidic acid biosynthesis	0	-0.01	0	0
tfaE	P09153	Phage or Prophage Related	-0.34	0.33	0.19	0.59
tfaS	P77326	Phage or Prophage Related	-0.29	0.33	0.24	0.67
tmk	P0A720	Enzyme; 2'-Deoxyribonucleotide metabolism	-0.51	-1.6	-0.43	-1.2
tpr	P02338	Factor; Basic proteins - synthesis, modification	-0.64	0.85	0	0.51
trkH	P0AFZ7	Transport; Transport of small molecules: Cations	-0.51	0.47	0	0
trxC	P0AGG4	Putative enzyme; Not classified	-0.75	0.45	0	0
uhpA	P0AGA6	Regulator; Transport of small molecules: Carbohydrates, organic acids, alcohols	-0.46	-0.16	-0.58	-0.44
uup	P43672	Factor; DNA - replication, repair, restriction/modification	0	0	-0.26	0.29
uxaA	P42604	Enzyme; Degradation of small molecules: Carbon compounds	-0.64	0.72	0	0.97
wbbI	P37749	Enzyme; Lipopolysaccharide biosynthesis	0	-1.27	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
wcaA	P77414	Putative regulator; Not classified	0.17	-0.89	0	0
wcaC	P71237	Putative enzyme; Not classified	-0.55	-1.26	-0.62	-0.21
wcaI	P32057	Putative enzyme; Not classified	-0.05	-0.06	0	0
wrbA	P0A8G6	Enzyme; Not classified	-0.47	-0.79	-0.72	-0.07
wzc	P76387	Enzyme; Not classified	-0.37	-0.07	0	0
xerC	P0A8P6	Enzyme; Cell division	0	0.13	-1.07	-0.67
yafM	Q47152	Null	-0.07	0.43	0	0
yagI	P77300	Phage or Prophage Related	-0.16	-0.68	-0.16	-0.16
yagU	P0AAA1	Null	1.1	-1.18	0	0
yaiV	P0AAP5	Null	0	-0.3	0	0
ybaY	P77717	Phenotype; Not classified	0.24	-0.12	0.01	-0.52
ybbA	P0A9T8	Putative transport; Not classified	0	-0.99	0	0
ybbN	P77395	Putative enzyme; Not classified	0	-0.1	0	0
ybcV	P77598	Phage or Prophage Related	-0.33	0.99	0.69	1
ybdN	P77216	Null	-0.76	0	0	0
ybdZ	P18393	Enzyme; Biosynthesis of cofactors, carriers: Enterochelin	0.15	0	0	0
ybeA	P0A8I8	Enzyme; rRNA modification	0	-0.04	0	0
ybeX	P0AE78	Putative transport; Not classified	-0.09	-0.1	0.54	-1.12
ybgJ	P0AAV4	Putative enzyme; Not classified	0	0.25	0	0
ybgO	P75748	Null	0	-1.53	0	0
ybiJ	P0AAX3	Null	-0.17	0.38	0	0
ybjH	P0AAY4	Null	0.62	-0.13	-0.75	-1.89
ybjI	P75809	Enzyme; Biosynthesis of cofactors, carriers: Riboflavin	0	0.34	0	0
ycbW	P75862	Null	0.17	-0.57	-0.22	-0.09
yccV	P0AB20	Null	0	-1.01	-0.63	-1.19
ycdN	P75901	Null	-0.43	0.08	0	0
yceK	P0AB31	Null	0	-0.44	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
ycgY	P76012	Null	-0.17	0	0	0
yciI	P0AB55	Null	-0.04	-0.6	-0.6	-0.65
yciQ	P45848	Null	-0.52	-1.65	0	0
ydaG	P76061	Phage or Prophage Related	0.12	-1.28	0	-1.98
ydaL	P76053	Null	0	-1.48	0.77	0.29
ydaT	P76064	Phage or Prophage Related	0.22	-1.97	-1.53	-0.96
ydaV	P77546	Phage or Prophage Related	0.48	0.28	-0.3	0.68
ydaW	P76066	Phage or Prophage Related	-0.13	-0.36	0.45	0.04
ydcI	P77171	Putative regulator; Not classified	0	-1.37	0	0
ydcQ	P67697	Null	-0.28	-0.56	0.45	0.26
ydcT	P77795	Putative transport; Not classified	0	0	-0.29	0
ydeA	P31122	Transport; Transport of small molecules: sugars	0	-2.08	0	0
ydeO	P76135	Regulator; Not classified	0	-0.7	0	0
ydfB	P29009	Phage or Prophage Related	-0.59	-0.78	-0.66	-0.69
ydhS	P77148	Putative enzyme; Not classified	-0.38	-1.07	1.06	0
ydiV	P76204	Null	0	-2.23	0	0
ydjN	P77529	Putative enzyme; Not classified	0.77	-1.13	0	0
yeaW	P0ABR7	Null	-0.08	0.33	0	0
yeaX	P76254	Putative enzyme; Not classified	0.1	1.03	0	0.93
yebU	P76273	Enzyme; rRNA modification	0	-1.74	0	0
yebV	P64503	Null	0	0.21	0	0
yecS	P0AFT2	Putative transport; Not classified	-1.22	0	0	0
yedQ	P76330	Null	-0.9	0.19	-1.34	-1.38
yedY	P76342	Putative enzyme; Not classified	-0.07	-0.98	0	-0.29
yeeO	P76352	Null	0.47	-0.47	0	-1.5
yeeY	P76369	Putative regulator; Not classified	-0.54	-1.99	0	0
yegI	P76393	Putative enzyme; Not classified	0	-0.15	0.15	-1.65

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
yegP	P76402	Null	0.03	-0.19	-0.18	-0.58
yegX	P76421	Null	-0.15	0	0	0
yehL	P33348	Null	0	-1.89	0	0
yehS	P33355	Null	-0.51	0	0	0
yfcU	P77196	Putative membrane; Not classified	0	-2.23	0	0
yfgG	P64545	Null	0	0.74	0.3	0.22
yfgJ	P76575	Null	0	-1.51	0	0
yfhR	P77538	Putative enzyme; Not classified	0.15	0.42	0	0
ygbJ	Q46888	Putative enzyme; Not classified	0	0.05	0	0
ygcS	Q46909	Putative transport; Not classified	0	0.08	0	0
ygdH	P0ADR8	Null	0.01	-0.85	-0.15	0.12
ygfM	P64557	Null	-0.05	-1.75	0	0
ygiC	P0ADT5	Putative enzyme; Not classified	0	-1.65	0	0
ygiL	P39834	Putative structural component; Surface structures	0	-0.03	0	0
ygjI	P42590	Orf; Not classified	0.64	0.21	1.07	1.07
ygjK	P42592	Enzyme; Not classified	0	0.03	0	0
yhbH	P0AFX0	Putative regulator; Global regulatory functions	0	-2.03	0	0
yhcG	P45423	Null	-0.76	0	0	0
yhcM	P64612	Enzyme; Not classified	0	-1.26	0	0
yhcO	P64616	Null	0.29	0.74	0	0
yhdH	P26646	Putative enzyme; Not classified	0	0.12	0	0
yhdX	P45767	Putative transport; Not classified	-0.88	0	0	0
yhgE	P45804	Putative transport; Not classified	0	-0.59	0	0
yhhW	P46852	Null	-0.3	-0.87	-1.16	-0.41
yhiQ	P68567	Enzyme; rRNA modification	-0.14	-0.37	-0.22	-1
yiaN	P37675	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	0	0.28	0.35	0
yidZ	P31463	Putative regulator; Not classified	-0.93	0.36	1.39	0.31

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
yifK	P27837	Putative transport; Not classified	-0.43	-1.11	-0.36	0.08
yihS	P32140	Enzyme; Not classified	-0.49	-0.51	0.11	-0.8
yijF	P32668	Null	0.44	0.37	0.44	0.31
yjaB	P09163	Null	0	0.77	0	0
yjaG	P32680	Null	-0.68	-0.89	-0.42	-0.6
yjaH	P32681	Null	-0.22	-0.63	-0.33	-0.62
yjcQ	P32715	Orf; Not classified	0	-0.51	0	0
yjfK	P39293	Null	0.19	-0.53	0	0
yjgK	P0AF96	Putative enzyme; Not classified	-0.12	-0.18	0.13	0.11
yjgM	P39337	Null	0	0.62	0	0
yjhF	P39357	Putative transport; Not classified	0	-0.77	-0.12	0.6
yjiN	P39385	Null	0.3	0.09	0.19	0.33
yjiR	P39389	Putative regulator; Not classified	-0.35	0.14	0	-1.69
yjjP	P0ADD5	Putative structure; Not classified	0	-0.58	0	0
yjjU	P39407	Null	-0.49	-0.36	0	0
ykfB	P77162	Phage or Prophage Related	0	-2.02	0	0
ykgF	P77536	Putative carrier; Energy metabolism, carbon: Electron transport	0.19	0	0	0
yliE	P75800	Null	0.04	-0.72	0	0
ymdB	P0A8D6	Null	-0.74	-1.28	0	0
ymdF	P56614	Null	0	-0.91	0	-0.59
ymfL	P75976	Phage or Prophage Related	0.17	-0.37	0.02	0.59
ymfR	P75979	Phage or Prophage Related	-0.71	-0.22	0	0
ymgF	P58034	Null	0	-0.09	0	0
yncB	P76113	Enzyme; Not classified	0	-1.22	-2.02	-0.68
yncC	P76114	Putative regulator; Not classified	-0.05	-0.97	0	0
yncE	P76116	Putative factor; Not classified	0.28	-0.43	0.29	0.85
yneF	P76147	Null	0.27	-1.53	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
yneI	P76149	Putative enzyme; Not classified	0.05	-0.39	0	0
yneK	P76150	Null	-0.53	0.17	0	0
ynfD	P76172	Null	-0.37	-0.78	-0.38	-1.39
ynhG	P76193	Enzyme; Murein sacculus, peptidoglycan	-0.18	-0.64	-0.57	-1.38
ynjB	P76223	Null	0	-0.49	0	0
ynjH	P76227	Null	-0.85	-0.57	0	0
yoeB	P69348	Null	-0.15	-1.06	0	0
yqfA	P67153	Putative enzyme; Not classified	0	-1.72	0	0
yqgB	P64567	Null	0.34	-0.17	-1.48	-1.07
yqhD	Q46856	Enzyme; Degradation of small molecules: Carbon compounds	0	-0.25	-0.2	-1.08
yqhG	Q46858	Null	-0.02	-0.77	-0.54	-0.36
yrfF	P45800	Putative factor; Not classified	1.19	-1.18	0	0
ytjC	P0A7A2	Enzyme; Not classified	0	-1.71	0	0
yaaA	P0A8I3	Null	-0.28	0.15	0.44	-1.54
zraR	P14375	Regulator; Energy metabolism, carbon: Fermentation	0.24	0.51	0.14	-1.66
Cluster 4						
apt	P69503	Enzyme; Salvage of nucleosides and nucleotides	0.51	1.96	1.71	0.53
asr	P36560	Phenotype; Not classified	0.77	2.22	0	0
cpxA	P0AE82	Putative regulator; Global regulatory functions	0.31	1.19	1.68	0.19
ddpX	P77790	Null	-0.43	1.42	0.4	0
fdnG	P24183	Enzyme; Energy metabolism, carbon: Anaerobic respiration	-0.37	0.91	0	0
hdeB	P0AET2	Null	0	1.43	0	-0.05
intF	P71298	Phage or Prophage Related	1.27	1.67	-0.7	0
menE	P37353	Enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	1.15	1.72	0	0
mhpR	P77569	Regulator; Not classified	-0.44	1.51	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
rarA	P0AAZ4	Putative enzyme; Not classified	1.07	1.99	0	0.43
rpmD	P0AG51	Structural component; Ribosomal proteins - synthesis, modification	-0.05	1.78	0	0.57
tldD	P0AGG8	Phenotype; Not classified	0.91	0.4	0.79	0.43
trg	P05704	Regulator; Chemotaxis and mobility	-0.12	2	0	0
yagZ	P0AAA3	Structural component; Surface structures	-0.22	1.15	0	0
yahI	P77624	Putative enzyme; Not classified	1.12	2.16	1.73	0
yahO	P75694	Null	0.71	2.39	0	0
ybaB	P0A8B5	Factor; DNA - replication, repair, restriction/modification	0.66	0.63	1.01	-0.07
ybjD	P75828	Null	-0.64	0.86	0	0
ycbV	P75860	Putative structural component; Surface structures	0.43	3.11	0	0
ycdP	P69432	Enzyme; Surface polysaccharides and antigens	-0.3	0.91	0	-0.57
ycjN	P76042	Putative transport; Not classified	-0.57	1.19	0	0
yeeA	P33011	Null	0.9	1.71	0	0
yeiJ	P33021	Putative transport; Not classified	0.31	1.55	0	0
yfcI	P77768	Null	-1.08	1.85	0	0
yggF	P21437	Null	1.51	2.13	0	0
ygiS	Q46863	Putative transport; Not classified	0	1.78	0	0
yhiN	P37631	Putative enzyme; Not classified	1.2	0.7	1.04	0.72
ykfI	P77692	Phage or Prophage Related	-0.53	1.24	0	0
ymfP	P75981	Phage or Prophage Related	0	1.94	0	0
yohD	P33366	Null	1.34	2.39	0	0
Cluster 6						
gapC	P33898	Null	0	-2.52	0	0
htrE	P33129	Putative membrane; Surface structures	0	-4.2	-0.54	-0.01
nagA	P0AF18	Enzyme; Central intermediary metabolism: Amino sugars	0	-3.25	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
secG	P0AG99	Transport; Protein, peptide secretion	0	-2.71	0	0
yfeX	P76536	Enzyme; Transport of small molecules: Cations	0	-2.7	0	0
yjiW	P39394	Null	0	-2.6	0	0
yqjK	Q47710	Null	0	-2.97	0	0
Cluster 7						
ccmD	P0ABM5	Transport; Protein, peptide secretion	-3.77	-1.75	0.45	0.25
cobS	P36561	Enzyme; Biosynthesis of cofactors, carriers: Cobalamin	-4.01	-3.6	-1.78	-0.61
nagC	P0AF20	Regulator; Central intermediary metabolism: Amino sugars	-3.09	-2.02	-0.41	-0.11
yfbT	P77625	Putative enzyme; Not classified	-2.52	-2.33	0	0
yfjZ	P52141	Phage or Prophage Related	-3.88	-3.16	0.96	1.19
Cluster 9						
cpsG	P24175	Enzyme; Central intermediary metabolism: Pool, multipurpose conversions	-1.3	-0.73	-0.45	0
csiR	P37338	Regulator; Not classified	-1.86	-0.94	-1.46	0
eutG	P76553	Putative enzyme; Degradation of small molecules: Amines	-1.14	0.26	-0.42	-0.97
fimC	P31697	Factor; Surface structures	-1.57	-0.58	-1.62	-0.18
flgD	P75936	Putative structure; Surface structures	-1.72	0.26	0.14	-0.85
hisH	P60595	Enzyme; Amino acid biosynthesis: Histidine	-0.84	-0.72	-0.15	-1.23
holC	P28905	Enzyme; DNA - replication, repair, restriction/modification	-1.96	0.27	-0.1	-0.97
hyfD	P77416	Enzyme; Energy metabolism, carbon: Anaerobic respiration	-1.87	-0.57	0	-1.18
imp	P31554	Phenotype; Adaptations, atypical conditions	-1.09	0.6	0	-0.78
lit	P11072	Membrane; Phage or Prophage Related	-2.72	0.59	0	0
mdlA	P77265	Null	-1.48	0	0	0
mraW	P60390	Enzyme; rRNA modification	-1.45	-0.86	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
mutT	P08337	Enzyme; 2'-Deoxyribonucleotide metabolism	-1.17	-0.54	0	-0.32
tar	P07017	Regulator; Chemotaxis and mobility	-1.38	-0.8	-1	0
thiS	O32583	Factor; Biosynthesis of cofactors, carriers: Thiamine	-1.19	-0.98	0	0
tktB	P33570	Enzyme; Central intermediary metabolism: Non-oxidative branch, pentose pathway	-1.98	-1.06	-0.43	-1.17
wzzE	P0AG00	Putative transport; Not classified	-1.6	-0.91	-0.76	0
yadD	P31665	Null	-1.19	-0.78	0	0
ybaV	P0AAR8	Null	-1.57	0	0	0
ycaO	P75838	Null	-0.47	0.41	-1.26	0.28
ycfM	P0AB38	Null	-1.38	-0.59	-0.73	-1.19
ydgD	P76176	Null	-1.21	0.35	-0.79	-0.15
yfbL	P76482	Putative enzyme; Not classified	-1.96	-0.32	0	0
ygiE	P0A8H3	Null	-1.17	0.31	-1.34	-0.11
yhbG	P0A9V1	Putative transport; Not classified	-2.22	-0.97	-1.12	0
yhcB	P0ADW3	Null	-1.14	-0.49	-0.48	-0.28
yiaT	P37681	Putative membrane; Not classified	-2.96	-0.99	0	-1.63
yidG	P0ADL6	Null	-2.19	-0.69	0.15	-1.03
yiiT	P0AAB8	Putative regulator; Not classified	-1.21	0.04	-0.27	-1.02
ymcD	P75885	Null	-2.36	0.49	-0.88	0
Cluster 10						
abgT	P46133	Putative transport; Not classified	0	1.66	3.35	3.74
ssnA	Q46812	Putative structure; Not classified	2.54	2.44	2.47	3
yfbG	P77398	Putative enzyme; Not classified	0.29	1.14	2.31	2.14

Table SVIII) Cluster containing genes with a low flux phenotype, ordered by 1% low flux value. Clusters derived from dendrogram in Figure SIII.

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
Cluster 2						
aceK	P11071	Enzyme; Central intermediary metabolism: Glyoxylate bypass	3.95	2.43	0	0
adhP	P39451	Enzyme; Energy metabolism, carbon: Anaerobic respiration	3.87	3.73	0	0
nanK	P45425	Putative regulator; Not classified	3.85	2.98	0	0
ybaL	P39830	Putative transport; Not classified	3.71	4	0	0
gatC	P69831	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	3.66	3.28	0	0
yedZ	P76343	Null	3.62	3.43	0	0
rimJ	P0A948	Enzyme; Ribosomes - maturation and modification	3.51	2.73	0	0
ppk	P0A7B1	Enzyme; Central intermediary metabolism: Phosphorus compounds	3.23	3.04	0.44	0
fliM	P06974	Structural component; Surface structures	3.22	2.01	0	0
yjjY	P0ADD9	Null	3.18	2.19	0	0
yafT	P77339	Putative enzyme; Not classified	3.17	2.21	0	0
ulaE	P39305	Putative enzyme; Central intermediary metabolism: Pool, multipurpose conversions	3.12	1.97	0.36	0.22
yfaO	P52006	Null	3.1	2.76	0	0
stfQ	P77515	Phage or Prophage Related	3.07	2.67	-0.17	0
stfR	P76072	Phage or Prophage Related	3.07	2.67	-0.17	0
flhB	P76299	Structural component; Not classified	3.07	2.18	0	0
yrbC	P0ADV7	Transport; Transport of small molecules: phospholipids	3.04	2.09	0	0
ynfN	P76157	Phage or Prophage Related	2.98	2.21	0.12	0.69
fliO	P22586	Putative structure; Surface structures	2.98	2.38	-0.48	0.01
ykgB	P75687	Null	2.95	2.91	0	0
pcm	P0A7A5	Enzyme; Proteins - translation and modification	2.95	2.69	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
dcuC	P0ABP3	Transport; Transport of small molecules: Carbohydrates, organic acids, alcohols	2.94	3.48	0	0.15
alsK	P32718	Putative regulator; Not classified	2.94	3.48	0	0.15
yhcE	P45421	Null	2.94	3.48	0	0.15
gatA	P69828	Enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols	2.94	3.48	0	0.15
phnC	P16677	Transport; Central intermediary metabolism: Phosphorus compounds	2.9	2.59	0	-1.23
fsaA	P78055	Null	2.88	2.45	0.58	0
yeaB	P43337	Null	2.84	2.7	-1.44	-0.59
paaI	P76084	Enzyme; Phenylacetic acid degradation	2.82	2.13	0.06	-1.09
ykfC	Q47688	Phage or Prophage Related	2.81	2.42	0	0
yncK	Null	Phage or Prophage Related	2.8	2.39	0	0
rssA	P0AFR0	Null	2.78	2.52	0	0
yagM	P71296	Phage or Prophage Related	2.75	2.09	0	0
dusB	P0ABT5	Enzyme; tRNA modification	2.74	2.12	0	-1.6
chbG	P37794	Enzyme; Degradation of small molecules: N,N'-diacetylchitobiose	2.62	2.31	0	0
ydeU	P32051	Null	2.6	1.99	0	0
uxuB	P39160	Enzyme; Degradation of small molecules: Carbon compounds	2.59	2.71	0	0
yagP	P75684	Putative regulator; Not classified	2.57	3.18	0	0
yfhD	P0AGC5	Enzyme; Transporter; Not classified	2.57	1.34	0	-0.98
ycfZ	P75961	Putative factor; Not classified	2.5	2.39	0	0
ynjI	P76228	Null	2.47	2.3	0	0
yeiB	P25747	Null	2.41	3.2	0	0
ycbF	P40876	Putative factor; Surface structures	2.28	2.26	0	0.63
dcp	P24171	Enzyme; Degradation of proteins, peptides, glyco	2.26	1.68	0	0
yoaB	P0AEB7	Putative enzyme; Not classified	2.21	1.87	0	0
flgK	P33235	Structural component; Surface structures	2.2	2.59	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
ybhP	P0AAW1	Null	2.12	2.05	-0.37	0
envY	P10805	Structural component; Outer membrane constituents	1.95	1.73	0	-1.46
hipA	P23874	Enzyme; Protein modification	1.72	1.45	-2.48	0
ymfO	P75980	Phage or Prophage Related	1.3	0.95	-1.6	0
flgI	P0A6S3	Putative structural component; Surface structures	0.76	0.24	-2.71	-0.56
yjgZ	P39351	Null	0.34	0.4	-1.91	0.87
Cluster 8						
mhpT	P77589	Putative transport; Not classified	2.76	2.54	-2.45	-2.38
mdfA	P0AEY8	Transport; Transport of small molecules: Other	2.48	1.86	-2.39	-3.38
ybaA	P0AAQ6	Null	0.93	1.44	-1.4	-1.65
ybdM	P77174	Null	0.73	0.45	-1.76	-2.66
galS	P25748	Regulator; Degradation of small molecules: Carbon compounds	0.28	2	-2.82	-0.86
galR	P03024	Regulator; Degradation of small molecules: Carbon compounds	-0.74	1.6	-2.14	-0.73
Cluster 3						
yjjG	P0A8Y1	Enzyme; Degradation of small molecules: Nucleotides	3.36	1.94	3.03	0.9
cheR	P07364	Enzyme; Chemotaxis and mobility	2.92	0.14	0	0
ydcR	P77730	Putative regulator; Not classified	2.89	1.42	0	0
ydfK	P76154	Phage or Prophage Related	2.55	1.55	0	0
ynaE	P76073	Phage or Prophage Related	2.55	1.55	0	0
ybeL	P0AAT9	Phenotype; Not classified	2.44	0.86	0	0
yafL	Q47151	Putative membrane; Not classified	2.43	1.46	0	0
ygcU	Q46911	Null	2.37	1.18	0	0
yfhB	P0AD42	Enzyme; Macromolecule synthesis, modification: Phospholipids	2.3	1.06	1.17	0.62
yjiM	P39384	Null	2.26	1.08	2.03	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
ilvB	P08142	Enzyme; Amino acid biosynthesis: Isoleucine, Valine	2.23	1.13	0.97	0
ulaF	P39306	Putative enzyme; Not classified	2.19	1.16	0	0
nfo	P0A6C1	Enzyme; Degradation of DNA	2.18	-1.09	0	0
cheW	P0A964	Regulator; Chemotaxis and mobility	2.02	1.15	0	0
ssuD	P80645	Null	1.94	0.95	-0.42	0
ykfF	P75677	Phage or Prophage Related	1.9	1.8	0	0
ybcD	P77528	Phage or Prophage Related	1.86	0.57	0	0
yaiF	P75697	Null	1.84	-0.84	0	0
yoeA	P76356	Phage or Prophage Related	1.84	-0.84	0	0
arpA	P23325	Null	1.82	1.67	1.94	0.11
mdlB	P0AAG5	Null	1.82	0.75	0.65	0.55
yafN	Q47156	Null	1.81	1.53	0	0
ydbA	P33666	Null	1.78	0.42	0	0
fliP	P0AC05	Putative structure; Surface structures	1.75	1.17	0	0
yfhK	P52101	Putative regulator; Not classified	1.74	0.86	0	0
nohA	P31061	Phage or Prophage Related	1.72	1.08	0	0
flgG	P0ABX5	Structural component; Surface structures	1.66	1.69	0.37	-0.31
mhpD	P77608	Enzyme; Degradation of small molecules: Carbon compounds	1.56	0.14	0.69	0
oppD	P76027	Transport; Not classified	1.5	0.85	0	0
ydeV	P77432	Enzyme; Not classified	1.5	1.14	0	0
hyfJ	P77453	Putative factor; Not classified	1.46	0.79	0	0
ymfM	P75977	Phage or Prophage Related	1.41	-0.13	-0.41	-0.42
aceB	P08997	Enzyme; Central intermediary metabolism: Glyoxylate bypass	1.36	0.35	0	0
ydiK	P0AFS7	Null	1.36	0	0	0
nagZ	P75949	Null	1.34	-0.71	0.65	0
ycdM	P75898	Enzyme; Pyrimidine nitrogen catabolism	1.34	0.76	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
ykgE	P77252	Putative carrier; Energy metabolism, carbon: Electron transport	1.32	1.15	0	0
yfdC	P37327	Putative transport; Not classified	1.29	-0.29	0	-0.45
glpD	P13035	Enzyme; Energy metabolism, carbon: Aerobic respiration	1.24	0.13	-0.36	-0.76
yahG	P77221	Null	1.23	0.96	0	0
ynaI	P0AEB5	Null	1.23	0.36	0	0
yahF	P77187	Putative enzyme; Not classified	1.22	0.36	-0.26	0
yahH	/	#N/A	1.21	0.83	0.64	-0.28
yfeO	P67729	Null	1.18	-0.59	0	0
asd	P0A9Q9	Enzyme; Amino acid biosynthesis: Lysine	1.13	0.95	-0.25	0
hyfR	P71229	Putative regulator; Not classified	1.11	-0.4	1.08	0.21
deoC	P0A6L0	Enzyme; Salvage of nucleosides and nucleotides	1.1	1.2	0.16	-0.7
yeiW	P0AFT8	Null	1.07	1.42	0.75	-1.26
yhcE	P45421	Null	1.03	0.29	0.34	0
yfgF	P77172	Enzyme; Not classified	1.02	-0.12	0	-0.63
fliS	P26608	Regulator; Surface structures	1.01	0.14	0	0
aceA	P0A9G6	Enzyme; Central intermediary metabolism: Glyoxylate bypass	0.98	1.31	0	0
ycbB	P22525	Enzyme; Murein sacculus, peptidoglycan	0.96	-0.22	0	0
ogt	P0AFH0	Enzyme; DNA - replication, repair, restriction/modification	0.95	0.14	0	0
yfiL	P11289	Null	0.95	0.58	0	0
cfa	P0A9H7	Enzyme; Fatty acid and phosphatidic acid biosynthesis	0.94	0.71	0	0
yihX	P0A8Y3	Enzyme; Not classified	0.92	-0.1	-0.3	0
yqgC	P64570	Null	0.92	1.33	0	0
yhaI	P64592	Putative carrier; Not classified	0.91	0.08	0	0
yccF	P0AB12	Null	0.89	1.18	0	0
yccX	P0AB65	Null	0.88	-0.5	0	0

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
rsuA	P0AA43	Enzyme; rRNA modification	0.86	0	0.07	0
hemE	P29680	Enzyme; Biosynthesis of cofactors, carriers: Heme, porphyrin	0.85	0.04	0.52	-0.3
purR	P0ACP7	Regulator; Purine ribonucleotide biosynthesis	0.83	0.81	-0.65	-0.61
yfdN	P76510	Phage or Prophage Related	0.83	0.82	0.17	-1.54
yneE	P76146	Null	0.82	0.82	-0.43	0.22
yafW	Q47684	Phage or Prophage Related	0.82	0.13	0	0
frdA	P00363	Enzyme; Energy metabolism, carbon: Anaerobic respiration	0.8	0	0	0
yeaP	P76245	Null	0.75	-0.08	0.61	-0.41
ycjW	P77615	Putative regulator; Not classified	0.73	0.09	0	0
yeaL	P0ACY6	Null	0.72	0.09	0	0
ycgH	Null	Phage or Prophage Related	0.7	0.55	-0.23	0.3
cynR	P27111	Regulator; Central intermediary metabolism: Pool, multipurpose conversions	0.7	1.24	0	0
pphA	P55798	Regulator; Not classified	0.67	0.66	0	-0.94
yjeK	P39280	Enzyme; Protein modification	0.66	0.44	0.4	-0.7
gatZ	P0C8J8	Putative enzyme; Not classified	0.6	0.57	0	0
kbaZ	P0C8K0	Putative enzyme; Not classified	0.59	-0.16	-0.1	0.11
ypfN	Q2EET2	Null	0.59	0.04	0.22	0
mrcB	P02919	Enzyme; Murein sacculus, peptidoglycan	0.58	-0.33	0.2	-0.35
ydeN	P77318	Putative enzyme; Not classified	0.57	0.09	0.17	-0.47
sgbU	P37679	Putative enzyme; Central intermediary metabolism: Pool, multipurpose conversions	0.57	-0.15	-0.08	-0.13
yecE	P37348	Null	0.56	-0.31	0.94	-0.2
kdpD	P21865	Enzyme; Global regulatory functions	0.55	0.96	0.25	-0.23
ilvN	P0ADF8	Enzyme; Amino acid biosynthesis: Isoleucine, Valine	0.54	-0.14	0	-0.32
atpB	P0AB98	Enzyme; ATP-proton motive force interconversion	0.54	0.16	0.56	-0.46
pntA	P07001	Enzyme; Central intermediary metabolism: Pool, multipurpose conversions	0.53	0.44	0	-0.66

Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
slyX	P0A8R4	Null	0.51	-0.06	0.08	0.32
atpG	P0ABA6	Enzyme; ATP-proton motive force interconversion	0.5	0.23	0.43	-1.02
ygaX	P76628	Putative transport; Not classified	0.47	-0.19	0	0
sucC	P0A836	Enzyme; Energy metabolism, carbon: TCA cycle	0.47	0.09	0.59	0.41
metJ	P0A8U6	Regulator; Amino acid biosynthesis: Methionine	0.45	-0.23	0.19	0.18
phnH	P16686	Enzyme; Central intermediary metabolism: Phosphorus compounds	0.41	-0.37	0	0
sfmC	P77249	Putative factor; Surface structures	0.41	0.62	0	-0.84
torS	P39453	Enzyme; Energy metabolism, carbon: Anaerobic respiration	0.36	-0.27	0	0
yfgH	P65290	Putative membrane; Not classified	0.3	-0.3	0	0

Table SIX) Cluster containing genes with a high flux phenotype, ordered by 5% high flux value. Clusters derived from dendrogram in Figure SIII. Genes that showed enrichment in both high flux pools and depletion in both low flux pools are highlighted

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Gene name	Locus tag	Function	1 % low flux	5 % low flux	1 % high flux	5 % high flux
Cluster 2						
ynfH	P76173	Putative enzyme; Degradation of small molecules: Carbon compounds	-2.12		1.35	2.35
rpiA	P0A7Z0	Enzyme; Central intermediary metabolism: Non-oxidative branch, pentose pathway	-2.24	-1.97	1.22	2.2
ompC	P06996	Membrane; Outer membrane constituents	-1.11	-0.72	1.02	1.26
tktA	P27302	Enzyme; Central intermediary metabolism: Non-oxidative branch, pentose pathway	-0.64	-0.27	0.35	1.17
tfaD	P77699	Phage or Prophage Related	-0.81	-0.06	-0.01	1.04
gst	P0A9D2	Enzyme; Biosynthesis of cofactors, carriers: Thioredoxin, glutaredoxin, glutathione	0.99	-0.65	3.55	0.82
cpdA	P0AEW4	Regulator; Degradation of small molecules: Carbon compounds	-0.23	0.61	2.53	0
hslV	P0A7B8	Enzyme; Degradation of proteins, peptides, glyco	-1.69	-2.59	0	0
ygbA	P25728	Null	-1.47	-1.24	0	0
yncH	P76118	Null	-1.46	-1.75	0	0
ypfG	P76559	Null	-1.26	-2.51	0	0
yfjM	P52128	Phage or Prophage Related	-1.92	-1.63	0	-0.1

Table SX) Growth rates of the *E. coli* BW25110 wild type strain, the 10 knockout strains that showed the highest enrichment in the low flux pools and the three knockout strains from the high flux analysis.

	Galactose		**Glucose**	
Deletion/ strain name	max growth rate (h^{-1})	OD_{600} [a)]	max growth rate (h^{-1})	OD 600 [b)]
Wild type	0.223 ± 0.006	0.14 ± 0.044	0.416 ± 0.052	0.49 ± 0.16
Low flux pool				
ybaL	0.245 ± 0.001	0.10 ± 0.001	0.336 ± 0.044	0.42 ± 0.25
yafT	0.231 ± 0.009	0.13 ± 0.01	0.37 ± 0.039	0.7 ± 0.24
yjjY	0.269 ± 0.017	0.09 ± 0.001	0.483 ± 0.094	0.67 ± 0.17
aceK	0.215 ± 0.009	0.13 ± 0.01	0.344 ± 0.028	0.84 ± 0.09
fliM	0.203 ± 0.021	0.10 ± 0.003	0.379 ± 0.014	0.75 ± 0.09
yedZ	0.214 ± 0.009	0.11 ± 0.004	0.416 ± 0.021	0.82 ± 0.13
gatC	0.216 ± 0.000	0.17 ± 0.06	0.38 ± 0.023	0.68 ± 0.21
nanK	0.213 ± 0.000	0.19 ± 0.21	0.366 ± 0.028	0.72 ± 0.2
adhP	0.221 ± 0.007	0.16 ± 0.02	0.409 ± 0.04	0.65 ± 0.23
rimJ	0.225 ± 0.002	0.11 ± 0.01	0.285 ± 0.064	0.54 ± 0.3
High flux pool				
ompC	0.356 ± 0.006	0.51 ± 0.07	0.295 ± 0.034	0.46 ± 0.24
rpiA	0.308 ± 0.005	0.85 ± 0.44	0.362 ± 0.007	0.16 ± 0.04
ynfH[c]	0.212 ± 0.007	0.13 ± 0.02	0.439 ± 0.035	0.64 ± 0.14

a) at 16 h
b) at 6.5 h

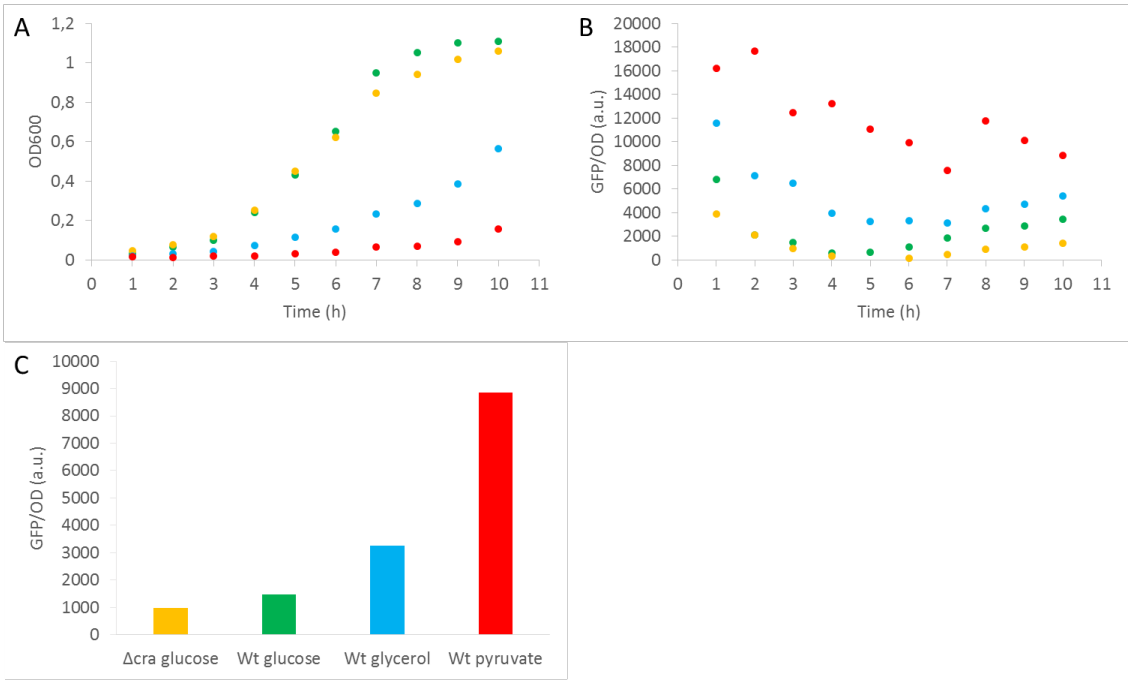
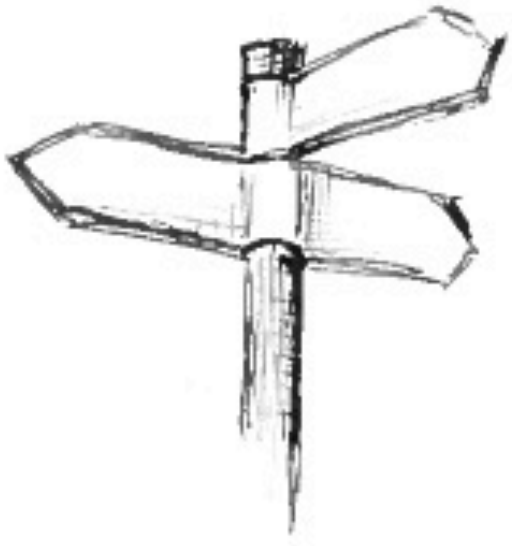


Figure S IV) **Analysis of growth and fluorescence over time in a plate reader.** To identify which time point is suitable for screening by FACS at a single time point, we analyzed the growth behavior and the GFP per OD in the wild type harboring pFlux growing in three different carbon sources (glucose: green, glycerol: blue and pyruvate: red) , and for the Δcra mutant harboring pFlux in glucose (orange). **(A)** Growth profile of the different strains. **(B)** Specific fluorescence given in GFP per OD (a.u.). **(C)** Specific fluorescence measured at OD600=0.13±0.02. n=2

CHAPTER 4 SUMMARY AND FUTURE PERSPECTIVES



4.1 Summary and conclusion

The aim of this study was to develop and characterize an improved glycolytic flux biosensor and apply it in a high-throughput screen to identify genes affected glycolytic flux in *E. coli*.

The glycolytic flux sensor is based on the *E. coli* transcription factor Cra, an idea originally introduced by Kochanowski *et al.* in 2013. In chapter 2, the design of an improved biosensor is described. The relative expression strengths of three native *E. coli* promoters (*pykF*, *ppc* and *ppsA)* controlling the expression of GFP were compared under different conditions. The expression levels of GFP when grown on glucose compared to galactose follow the expected tendencies of a glycolytic flux reporter. It is expected that the signal intensity of the activated promoter of *ppsA* increases with decreasing flux whereas the signal intensity of the two repressed promoters *pykF* and *ppc* decreases with decreasing flux. Interestingly, the growth on the gluconeogenic carbon source pyruvate resulted in the highest fluorescent signal for all three cases. There is experimental evidence for the mode of action of the different promoters (Bledig *et al*, 1996b; Nègre *et al*, 1998; Shimada *et al*, 2011b), proving that the highest values of pykF and ppc promoters in pyruvate must have another reason. It can be assumed that this observed effect correlates with the highly different growth rates between glycolytic and gluconeogenic carbon sources (Klumpp *et al*, 2009). During bacterial cell division, the intracellular fluorescent protein concentration is diluted. If a bacterial cell is growing fast, the *gfp* production is not fast enough to compensate for this effect. In contrast, if the cells are growing slow there is more time to accumulate *gfp* in the cells. This observed effect made comparison of the promoters more difficult, as there was a definite bias in the data. Nevertheless, *ppsA* appeared to be the most interesting candidate to proceed further, as it showed the by far highest dynamic range additionally to a comparably low expression in the OFF state (Glucose) (Figure 2.1B).

The effects of growth rates and possible other environmental effects emphasized the necessity of an internal reference to create a biosensor with a reliable single cell output. Kochanowski et al. applied an external control in which they compared the fluorescent expression from a promoter with a native and a scrambled Cra binding site. This approach was combined with a two-colour screening method, which has been previously shown by Kosuri

and co-workers to be a reliable setup for FACS experiments (Kosuri *et al*, 2013), for the construction of our final flux sensing plasmid (pFlux).

pFlux was tested in different *E. coli* strains on gluconeogenic carbon sources, additionally to the glycolytic carbon sources that were previously described in the study of Kochanowski *et al*. The presented findings (Figure 2.3) give a clear and consistent correlation between different carbon sources, inducing different glycolytic fluxes, and Cra activity, monitored via the fluorescence signal of the flux biosensor. It was furthermore possible to show that the screening capabilities of pFlux exceed the normal, physiological range by using it to measure the increased glycolytic flux during mevalonate production from pMevT (Martin *et al*, 2003). This characteristic of pFlux can be of high interest for further applications of this biosensor in metabolic engineering, as an increase in total flux could be used to increase the flux in the production pathway as well (LaCroix *et al*, 2015).

The presented findings in chapter 2 also support the claims of Kochanowski *et al.* that Cra functions as a direct glycolytic flux sensor in *E. coli*, even though it has different functions in other organism (Chavarría *et al*, 2014). Nevertheless it needs to be noted that Cra, and consequently pFlux, have limitations in the capabilities as flux sensors. We could show that the growth on fructose resulted in the lowest signal ratio of GFP to RFP (Figure 2.3), even though fructose should not induce the highest glycolytic flux. However, this finding does not come unexpected, as the affinity of Cra is actually higher for F1P, an intracellular metabolite of fructose metabolism, than FBP (Ramseier *et al*, 1993). Accordingly, it emphasises that the Cra activity is actually controlled by the dynamic equilibrium between Cra, FBP and F1P and not only the interaction between FBP and Cra. In most cases, the intracellular concentration of F1P is negligible and the correlation between FBP and glycolytic flux can be assumed (Kochanowski *et al*, 2013). Nevertheless, it is important to keep this finding in mind when applying pFlux.

Chapter 3 regards the application of pFlux in a large-scale physiological study of *E. coli* knockout mutants and their effect on the glycolytic flux. The aim is to use pFlux to enable rapid identification of gene knockouts or potentially other genetic variants that affect glycolytic flux in *E. coli*. Currently, it is not possible to obtain information about metabolic fluxes in high throughput. Techniques such as ^{13}C flux analysis have high resolution and give detailed information about the whole flux network, but are time consuming and thus the throughput only allows screening of individually selected mutants (Fischer *et al*, 2004; Crown *et al*, 2015).

Depending on the application, established techniques give high-resolution information of different fluxes (Leighty & Antoniewicz, 2013) in individual strains, whereas the Cra-based biosensor provides an opportunity to screen, compare and sort up to a theoretical 10^5 different variants per second, limited by the current physical screening capacities of modern flow cytometers and FACS instruments.

In this study we have successfully screened a library of 2,126 *E. coli* deletion mutants of the KEIO collection for their effects on the glycolytic flux when grown on galactose. The Δ*rpiA* strain was one of the rare examples with a high flux phenotype and is furthermore a very unexpected candidate for the increase of glycolytic flux. After further research, it appears that these effects might correlate to an altered expression of RpiB, which has thus far been considered a rather irrelevant enzyme (Sørensen & Hove-Jensen, 1996). In another more recent study though, it already appeared that RpiB might have a larger impact on the metabolism of E. coli as so far anticipated (Kim & Reed, 2012), which is supported by our findings.

In regard to the genes with a low flux phenotype, certain genes could be directly linked to the galactose metabolism (*galS*, *galR*), which is a good indication that the allocation of the genes in low and high flux pools correlates with the actual low and high flux state of the cells. Additionally to the expected galactose metabolism genes, it could be shown that the deletion of flagella related genes as well as many phage and prophage genes and genes with so far unknown function result in a decreased glycolytic flux. As those deletion strains were not outcompeted by the strains with little to no flux changes, the knockout of these genes seemed not to cause a severe fitness defect, even though they had a decreased flux. This characteristic of a low flux phenotype while maintaining a competitive fitness makes those strains highly interesting for possible production strains, since those strains may have a lower metabolic maintenance requirement compared to the wild type potentially enabling the direction of more flux towards production objectives.

To summarize, the most important improvement of the here presented pFlux biosensor is that it can be applied in flow cytometry and FACS experiments. This enables fast, easy, cheap and parallelizable high-throughput comparisons of glycolytic fluxes. Furthermore, the application of pFlux in an explorative library screen successfully identified possible genetic targets in order to improve future production strains.

4.2 Outlook

In this PhD study the fully functional biosensor pFlux was developed, tested and applied to study the effects of specific gene knockouts on the glycolytic flux in *E. coli*. An initial test in combination with a production pathway and its application in a FlowSeq experiment gave a first glimpse in the vast amount of possible applications pFlux can be used for.

Based on the presented research in chapter 2, the next steps in order to establish pFlux, would be to apply it in an actual metabolic engineering approach, possibly for the further development of the mevalonate pathway. More interestingly though, would be the integration of the Cra biosensor together with a product sensor in a synthetic regulation circuit.

Initial steps have been taken in order to create a selection system based on Cra, which could facilitate elaborated control circuits, which could for example be applied in production processes. By detecting as well the state of the glycolytic flux as the expression level of a target molecule, it could be possible to delete cells with no production but high growth rate from the process, limiting the chances that the fermentation will be overgrown by bacteria that escaped the production (Lieder *et al*, 2014; Delvigne & Goffin, 2014). It has been shown for several different species and as well for the production of small molecules as for the expression of whole proteins, that production strains tend to exit the production.

One of the main advantages of pFlux is that it is not product or pathway specific. The applications of most current biotechnologically relevant biosensors focus on improving the production of one specific target or a group of structurally related targets like for example fatty acids (Xu *et al*, 2014). pFlux, however, is highly versatile as it can be applied in any study that targets or involves the glycolytic flux in *E. coli*. Recent research on host orthogonality proved the possibility to not only successfully transfer repressors, but also activators from *E. coli* to yeast (Skjoedt *et al*, 2016). Especially due to the application versatility of Cra, it could be a very interesting candidate for further developments in yeast, also because no similar regulation mechanism is known yet in yeast.

In regards to chapter 3, the next step will be to apply the identified mutants for actual expression pathways in order to validate the increase in yield or productivity. A very promising candidate would be the deletion of flagella, as this production of flagella is very costly and not necessary in a stirred fermenter. It will be interesting to see, whether the lower house keeping demand will actually result in a higher production phenotype. Additionally, the Δ*rpiA* mutant

requires further studies in order to explore the causes for the very and unexpected high growth rates. A sequence analysis of the Δ*rpiA* genome could reveal mutations and possibly support the hypothesis that RpiB plays a way more important role than thus far expected (Sørensen & Hove-Jensen, 1996; Kim & Reed, 2012).

Another logical follow-up experiment would be the screening of an overexpression library as for example the ASKA collection (Kitagawa *et al*, 2006), in order to see how those effect the glycolytic flux. Besides finding other possible candidates for biotechnology, it would be highly interesting to see the correlations between the effects when a gene is knocked out compared to being overexpressed. Matching both data sets could help to decide which of the thus far unknown genes should be further studied.

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Scientific publications and dissemination

As a result of this PhD project, the following scientific publications are in preparation.

1.) Lehning, CE; Siedler, S; Cardinale, S; Ellabaan, MMH; Sommer, MOA (2016). Glycolytic flux sensing at the single cell level: genome wide identification of flux changing genes in *E. coli* (manuscript in preparation) *)

2.) van der Helm, E; Bech, R; Lehning, CE, Sommer, MOA (2016). Profiling bacterial kinase activity using a genetic circuit (manuscript in preparation) **)

The results of the studies presented in chapter 2 and 3 were presented in form of poster and oral presentations at the following instances.

Poster presentations

- 1st Systems and Synthetic Biology Summer School; 2014
- DMS Congress; 2014
- CFB annual seminar; 2015

Oral presentations

- 2nd PROMYS project meeting
- 3rd PROMYS project meeting
- 5th PROMYS project meeting

*) Comprises the results of chapter 2 and 3.
**) Only minor contributions; results from this publication are not considered in this thesis.